

**ISOLATION AND MOLECULAR
CHARACTERISATION OF TWO *Pseudomonas* sp.
ACC DEAMINASE GENES**

BRIDGET GENEVIEVE CAMPBELL

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**Isolation and molecular characterisation of two
Pseudomonas sp. ACC deaminase genes**

Bridget Genevieve Campbell

A dissertation submitted in fulfilment of the requirements for the degree of Master
of Science in the Faculty of Science, University of Cape Town.

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Isolation and Molecular Characterisation of two *Pseudomonas* sp. ACC Deaminase Genes

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ABSTRACT

The phytohormone ethylene is essential to many plant developmental processes, of which the control of climacteric fruit ripening is among the best characterised. However this hormone eventually causes fruit rotting which results in a non-marketable product. One approach to reduce ethylene synthesis in plants is metabolism of its immediate precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). This can be achieved through degradation of ACC by the enzyme ACC deaminase to form α -ketobutyric acid and ammonia.

ACC degrading soil microorganisms were identified by their ability to grow on ACC as a sole nitrogen source. Enzyme assays indicated that *Pseudomonads* had high ACC deaminase activity whereas yeasts had low activity. PCR and Southern blot analysis with ACC deaminase gene-specific primers and probes respectively revealed that only one bacterium, *Pseudomonas fluorescens* strain 17, had a gene with homology to previously sequenced ACC deaminase genes.

Genomic DNA libraries of two *P. fluorescens* strains displaying the highest ACC deaminase activity (strains 15 and 17) were constructed in the suicide vector pEcoR251. Screening the libraries on minimal medium containing ACC as the sole nitrogen source resulted in the isolation of plasmids conferring ACC degrading ability. One plasmid from each library, pBC15 (strain 15) and pBC17 (strain 17), was chosen for further studies.

Sequencing a 1.9-kb *PvuII* subclone of pBC17 indicated that the *P. fluorescens* strain 17 ACC deaminase gene was highly homologous to previously sequenced genes. It showed 86.8% similarity to the gene from *Pseudomonas* sp. strain 65G, and 71.7% homology to that of *Pseudomonas* sp. strain ACP. Preliminary end sequencing studies of pBC15 subclones confirmed PCR and Southern blot analyses which suggested that the *P. fluorescens* strain 15 ACC deaminase gene is very different to previously sequenced genes.

The *P. fluorescens* strain 17 ACC deaminase gene was PCR amplified and cloned into an *A. tumefaciens* Ti-plasmid-based binary vector. *A. tumefaciens* was transformed with recombinant plasmids. These strains are now ready for transfer into pome and stone fruit plants to generate transgenic fruit with potentially delayed ripening and thus a longer shelf life.

CHAPTER 1

Literature Review: The Molecular Biology and Control of Fruit Ripening

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CHAPTER 1

Literature review: The molecular biology and control of fruit ripening

1.1 INTRODUCTION

Many flowering plants invest large amounts of energy in the production of fleshy fruits in order to promote the dispersal of their seeds. During the final stages of development, fruit undergo a complex series of biochemical and physiological events involving changes in colour, flavour, aroma, texture and nutritional status that make them both attractive and tasty to eat. These ripening processes are due to alterations in the activity of several biochemical pathways, and involve all cell compartments.

The ripening programme requires differential gene expression and is modulated by environmental conditions and phytohormones (plant growth regulators). The role of the plant hormone ethylene in fruit ripening is well established (Klee *et al.* 1991). Chemical inhibitors of ethylene synthesis or action completely block ripening of fruits in many plant species (Grierson *et al.* 1986a). A number of tomato fruit ripening mutants, such as Neverripe (*Nr*) and ripening inhibitor (*rin*) have also been identified (DellaPenna *et al.* 1989; Picton *et al.* 1993a; Lanahan *et al.* 1994). These mutants do not respond to ethylene and are unable to ripen. At the molecular level, ethylene is known to induce expression of a number of genes involved in ripening (Lincoln and Fischer 1988).

In this chapter I will highlight the importance of the role of ethylene in the ripening phase of climacteric fruits. Firstly I will outline the role of ethylene in ripening, and describe the biosynthesis of ethylene in higher plants. At this point I will briefly compare plant and microbial ethylene biosynthetic pathways before discussing the alterations in colour, flavour and texture during ripening. The above information will highlight the use of antisense genes in manipulating fruit ripening. I will conclude

this chapter by outlining the significance and advantages of using the bacterial 1-aminocyclopropane-1-carboxylic (ACC) acid deaminase gene to control ripening.

1.2 RIPENING - THE FINAL PHASE IN FRUIT DEVELOPMENT

Ripening is an aspect of development that is unique to fruit, and is initiated after the completion of seed maturation. Ripening is defined as changes that occur in fruit from the latter stages of growth and development, through the early stages of senescence, and results in characteristic aesthetic and/or food quality (Brady 1987).

In most plants early fruit development can be divided into three phases (Gillapsy *et al.* 1993). The earliest phase involves the development of the ovary and the decision to abort, or to proceed with further cell division and fruit development. In the second phase, fruit growth can be primarily be attributed to cell division. The third phase begins after cell division ceases. During this phase fruit growth continues, mostly by cell expansion, until the fruit reaches its final size. Phytohormones play an important role in all three phases of development.

At the end of the third phase of development the fruit is firm, fleshy, and green in colour. There is now an increase in the production of the phytohormone ethylene, otherwise known as the ripening hormone. This plant growth regulator stimulates the expression of specific ripening genes, which affect colour, flavour, texture and aroma. These changes make the fruit more attractive to eat.

1.3 THE ROLE OF ETHYLENE IN RIPENING

People have inadvertently recognised the role of ethylene in fruit ripening for many years. Folklore states that ripening fruit in sealed bags hastens the ripening process. This is true as sealed bags trap ethylene produced by the fruit, and ethylene enhances fruit ripening. The earliest record of human manipulation of ripening is in the old Testament where Amos describes himself as a piercer of sycamore fig fruit. Later the

Greek philosopher Theophrastus noted that sycamore fig fruits do not ripen unless scraped with an iron claw. Twenty three centuries later it was noted that wounding induces ethylene production which in turn stimulates the ripening process. In 1924 Denny identified ethylene as the component in combustion fumes from kerosene stoves that caused lemon degreening, and described it as a ripening reagent (Theologis 1992).

Fruits can be divided into two classes, viz. climacteric and nonclimacteric, based on their pattern of respiration and response to ethylene during maturation and ripening. In climacteric fruit, application of exogenous ethylene hastens the respiratory peak and ripening associated changes (McGlasson 1971). However, nonclimacteric fruit ripen without an accompanying increase in respiration or ethylene synthesis. Nonclimacteric fruits have also been classified as fruits whose respiratory pattern shows a slow drift downwards after detachment from the parent plant (Rhodes 1970).

There are several lines of evidence which implicate ethylene as being the major component in the ripening of climacteric fruit. Firstly, the application of exogenous ethylene to unripe fruits hastens the ripening process (Oeller *et al.* 1991). Furthermore, initiation of ripening in climacteric fruits is marked by a sharp rise in ethylene production (Gray *et al.* 1992). Transgenic plants with antisense constructs to one of the enzymes involved in ethylene biosynthesis also fail to ripen, or ripen at a slower rate (Oeller *et al.* 1991; Picton *et al.* 1993b). These fruits ripen when exogenous ethylene is applied. Thus it can be seen that ethylene plays an important role in initiating ripening in climacteric fruits.

Another line of evidence that implicates ethylene in fruit ripening is the use of chemicals to inhibit ethylene's synthesis or mode of action. Application of aminoethoxyvinyl glycine (AVG) retards the ripening process by targeting an enzyme, ACC synthase, which is involved solely in ethylene biosynthesis (Bufler 1984). The inhibition of ripening by AVG is overcome by subsequent exposures to ethylene. Furthermore, silver ions also inhibit ripening by interfering with the plant's ability to detect or respond to ethylene (Grierson *et al.* 1986).

1.3.1 The role of ethylene in other plant functions

Although ethylene clearly plays an important role in initiating fruit ripening, this phytohormone is also involved in regulating a number of other plant processes. Ethylene promotes abscission of leaves, flowers and fruits by triggering the synthesis of enzymes that cause the cell wall dissolution associated with abscission (Raven *et al.* 1986). Ethylene promotes germination via an increase in aerobic respiration, which influences the water potential of the seed (Cerevantes *et al.* 1994). This phytohormone also regulates flowering and senescence of plants.

Ethylene is stimulated in response to environmental stresses such as mechanical wounding, waterlogging and pathogen attack by bacteria and fungi. Following pathogen attack, combinations of the ethylene and methyl jasmonate have been shown to synergistically induce members of the pathogenesis related superfamily of defence genes in tobacco (Xu *et al.* 1994). Balague *et al.* (1993) demonstrated that expression of ethylene forming enzyme (ACC oxidase) was induced following wounding. Similar results were obtained by Lincoln *et al.* (1993) for ACC synthase, another enzyme involved solely in the ethylene biosynthetic pathway. Enyedi *et al.* (1992) proposed that ethylene is synthesised in response to mechanical or insect wounding, which often facilitates pathogen invasion. Thus an increase in ethylene in response to wounding would induce the expression of pathogenesis related proteins in an attempt to stop the spread of disease in plants.

1.3.2 The mode of action of ethylene

Ethylene exerts its control by modulating the expression of specific genes involved in the changes in flavour, colour and texture which take place during ripening. In contrast with the wealth of information available on biosynthesis of ethylene, little is known about the molecular mechanisms involved in perception and subsequent transduction of this plant hormone signal. However, the identification of *Arabidopsis thaliana* mutants that are affected in their response to ethylene has helped to elucidate the ethylene signal transduction pathway (Theologis 1993).

Three *Arabidopsis* proteins are believed to play a role in ethylene signal transduction

viz. EIN1, CTR1 and EIN3 (Theologis 1993). Analysis of mutants suggest that the proteins act in a signalling pathway in the order: EIN1- CTR1- EIN3. EIN1 is thought to be the signal sensor, CTR1 the signal transducer, and EIN3 is a signal receiver. Additional components in this pathway have been identified but their exact function and role is still unclear (Zarembinski and Theologis 1994). ETO1 is a protein that is a negative regulator of ethylene biosynthesis, probably at the level of ACC synthase gene expression. *Arabidopsis* mutants have also identified AIN1 as a protein involved in ethylene biosynthesis. This protein has been shown to positively regulate ethylene production (Zarembinski and Theologis 1994).

EIN1, the ethylene signal sensor, is believed to be a Zn^{2+} or Cu^+ containing metalloprotein (Burg and Burg 1967). It is of great interest, therefore, that tomato seedlings deficient in Zn^{2+} do not respond to ethylene (Theologis 1993). However, attempts to isolate the putative metalloprotein receptor for ethylene (EIN1) have been unsuccessful.

Kieber *et al.* (1993) have cloned the CTR1 gene. This is the first component of the ethylene signal transduction pathway to be characterised. CTR1 encodes a 92kD putative serine/threonine kinase, which is related by sequence to the Raf protein kinase family (Kieber *et al.* 1993). Cytosolic Raf protein kinases are responsible for signal integrating enzymes that communicate between multiple cell surface receptor systems and the nucleus in animal cells (Theologis 1993).

Raf-1 activation in human cells is through direct phosphorylation of Raf-1 by growth factor receptors with intrinsic tyrosine kinase activity (Mamon *et al.* 1991). Based on this information, Theologis (1993) has proposed a biochemical model for the ethylene signal transduction pathway in plants. EIN1, a probable metalloprotein receptor, is active in the absence of ethylene and inactive in its presence. Activated EIN1 is the kinase responsible for the phosphorylation and activation of CTR1 in the absence of ethylene. Activated CTR1 may phosphorylate EIN3. This is probably a transcription factor that is inactivated by phosphorylation. Therefore in the presence of ethylene, EIN1 would not be activated and thus CTR1 would not be phosphorylated. This

inactive form of CTR1 can not phosphorylate EIN3. EIN3 would then be active, allowing transcription of new gene products involved in changes in colour, texture and flavour which occur during fruit ripening.

1.3.3 The Respiratory Climacteric

In climacteric fruit the onset of ripening is accompanied by an increase in respiration, a phenomenon known as the respiratory climacteric. This burst of respiration may be necessary to the fuel changes which occur during ripening of climacteric fruit. The onset of the respiratory increase is stimulated by the phytohormone ethylene, and the respiratory climacteric is accompanied by a peak in ethylene synthesis (Gray *et al.* 1992).

Rhodes (1970) defined the respiratory climacteric as a period in the ontogeny of certain fruits during which a series of biochemical changes is initiated by the autocatalytic production of ethylene. This phase marks the change from growth to senescence, and involves an increase in respiration which leads to ripening. Changes in respiration during ripening differ between fruit species with a 60-100% rise being found in apples at normal ripening temperatures (Rhodes 1970). Tomatoes and bananas exhibit a three-fold respiratory rise, whereas avocados show a four- to five-fold rise. In the tomato and the apple, the rise in respiration occurs in fruit both attached to and detached from the vine. However the avocado only shows a climacteric rise in respiration after detachment from the tree (Rhodes 1970).

In many tropical and subtropical fruits the rise in respiration is rapid, and the stage of eating corresponds closely with the climacteric peak. Examples of such fruits are avocado, banana and mango. On the otherhand, fruits such as the apple and tomato do not ripen completely until some time after the climacteric peak. Thus in tropical fruits the various changes involved in the climacteric and ripening show a more complete overlap time (Rhodes 1970).

1.3.4 Climacteric and Non-Climacteric Fruits

As mentioned previously, fruits can be divided into climacteric and non-climacteric

classes. Most species of fruit belong to the first class and undergo a distinct ripening phase. Examples of climacteric fruits include apples, bananas, pears, melons, avocados and tomatoes. Members of the non-climacteric class of fruits are grapes, strawberries, citrus and pineapple. These fruits have a well defined progression of ripening characterised by defined stages. However these stages are not correlated with a well defined peak of ethylene production or respiration. At least some new enzymes are synthesised during ripening of non-climacteric fruits indicating that changes in gene expression are involved in this process (Manning 1994).

Manning (1994) examined the changes during the ripening of strawberries, a non climacteric fruit. Strawberry fruit development is characterised by gross changes in protein and total RNA levels in receptacle tissue, as well as the more obvious visual changes in chlorophyll and anthocyanin pigmentation. Unlike climacteric fruits, the phytohormone auxin was found to regulate changes in gene expression during strawberry fruit ripening (Knee *et al.* 1977; Manning 1994). However, auxin is a repressor of ripening in the strawberry, whereas ethylene is an inducer of ripening in climacteric fruit. Both these hormones coordinate a series of changes in gene expression during this important phase of fruit development. It has not yet been shown that auxin plays a role in the ripening of other non climacteric fruits. Unlike climacteric fruits where ethylene is the common ripening factor, non-climacteric fruit species all appear to differ with respect to their mechanism of ripening (McGlasson 1970).

1.4 THE BIOSYNTHESIS OF ETHYLENE

Ethylene is one of the simplest organic molecules with biological activity. Because of its commercial importance and profound effects on plant growth, the biosynthesis of ethylene and mechanisms of its action have been intensely investigated. Ethylene is produced in plant tissues in amounts ranging from none to 500 nl/g of plant tissue per hour. This gaseous phytohormone is biologically active in trace amounts of 10 to 100 nl/l air (Theologis 1993).

Yang and Hoffman (1984) were the first biochemists to outline the mechanism of ethylene biosynthesis in higher plants. S-adenosyl transferase catalyses the conversion of methionine to s-adenosylmethione (SAM). SAM in turn is converted by the enzyme ACC synthase to methylthioadenosine (MTA) and 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene. This is the rate limiting step in ethylene's biosynthesis (Theologis 1992). ACC is oxidised to CO₂, HCN and ethylene by a complex enzymatic reaction catalysed by ACC oxidase, otherwise known as ethylene forming enzyme. The ethylene biosynthetic pathway (the Yang Cycle) is diagrammatically represented in Figure 1.1.

Because the level of methionine in cells is too low to sustain the normal rate of ethylene production, methionine must be recycled (Yang and Hoffman 1984). This is achieved by recycling MTA to methionine (see Figure 1). The overall result is that the ribose moiety of ATP gives rise to the 4 carbon skeleton from which ethylene is derived (Theologis 1992). In this manner the methylthiol group is conserved for continued regeneration of methionine. Thus with a constant pool of methylthiol groups and available ATP, high rates of ethylene production can be achieved.

No ethylene biosynthetic enzymes have been purified to homogeneity because of their low abundance in plant tissue (Theologis 1992). The cloning of genes coding for ACC synthase and ACC oxidase was a prerequisite for the study of regulation of ethylene biosynthesis at the molecular level (Gomez-Lim *et al.* 1993). Molecular cloning and heterologous expression of genes has allowed the isolation and subsequent characterisation of genes encoding s-adenosyltransferase (Peleman *et al.* 1989), ACC synthase (Rottman *et al.* 1991), and ACC oxidase (Hamilton *et al.* 1990).

1.4.1 ACC Synthase

After the discovery that ACC is the immediate precursor of ethylene, it became obvious that the enzyme whose activity limits ethylene biosynthesis is ACC synthase (s-adenosyl-L-methionine methylthioadenosine-lyase) (Yang and Hoffman 1984). Induction of ethylene production requires *de novo* synthesis of this enzyme (Kende 1989). ACC synthase is induced by conditions that cause ethylene biosynthesis, viz.

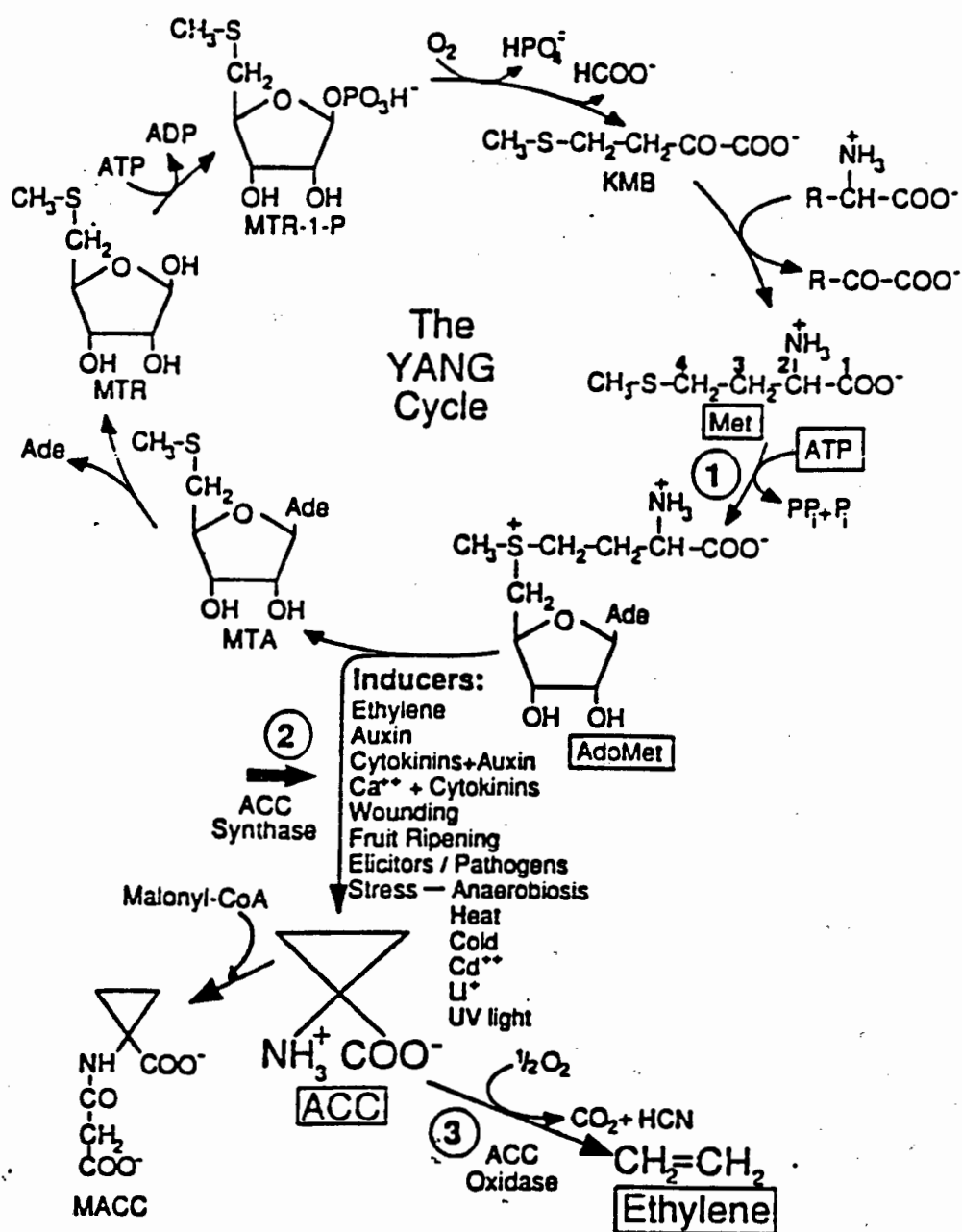


Figure 1.1 The ethylene biosynthetic pathway in higher plants

AdoMet, S-adenosyl-L-methionine; ACC, 1-aminocyclopropane-1-carboxylic-acid; KMB, 2-keto-4-methylthiobutyrate; MACC, malonyl-ACC; MTA, 5-methylthioadenosine; MTR, 5-methylthioribose; MTR-1-P, MTR-1-phosphate (Yang and Hoffman 1984).

Figure 1.1 The ethylene biosynthetic pathway in higher plants

AdoMet, S-adenosyl-L-methionine; ACC, 1-aminocyclopropane-1-carboxylic-acid; KMB, 2-keto-4-methylthiobutyrate; MACC, malonyl-ACC; MTA, 5'-methylthioadenosine; MTR, 5'-methylthioribose; MTR-1-P, MTR-1-phosphate (Yang and Hoffman 1984).

mechanical wounding (cutting, bruising and insect infestation), temperature change (freezing, chilling, radiation and occasionally high temperatures), drought, flooding and chemicals (Lincoln *et al.* 1993). The induction of this enzyme is due to an increase in accumulation of ACC synthase mRNA (Sato and Theologis 1989; Dong *et al.* 1991).

A fundamental question arose as to whether there are as many ACC synthase genes as inducers, or if there is only one gene whose expression is somehow activated by all of the inducers (Lincoln *et al.* 1993). Isolation of ACC synthase mRNA and subsequent characterisation of cDNA clones from different plant species have helped to answer this question.

ACC synthase genes have been cloned from a number of plants, and experimental evidence indicates that the enzyme is encoded by a highly divergent multigene family. As many as six ACC synthase genes have been identified in tomato (Lincoln *et al.* 1993; Rottman *et al.* 1991), three in mung beans (Botella *et al.* 1993), two in zucchini (Huang *et al.* 1991) and five in *A. thaliana* (Liang *et al.* 1992). ACC synthase genes have also been cloned from apples (Dong *et al.* 1991; Ross *et al.* 1992). Comparison of the structure and expression of twenty ACC synthase genes suggests that extensive polymorphism and distinct regulatory networks governing the expression of ACC synthase subfamilies arose early in plant evolution, prior to the divergence of monocots and dicots (Liang *et al.* 1992; Theologis 1992).

Examination of the patterns of gene expression of different members of an ACC synthase multigene family has indicated that they are differentially responsive to various ethylene inducing factors, although there is some level of coordination (Dong *et al.* 1991; Rottman *et al.* 1991; Van Der Straeten *et al.* 1991; Rodrigues-Pousada *et al.* 1993). For example, Lincoln *et al.* (1993) showed that two tomato ACC synthase genes, LE-ACS2 and LE-ACS4, are expressed in ripening fruits and in response to wounding. However, the wound induced accumulation of LE-ACS2 is more rapid and greater than that of LE-ACS4. The promoters of LE-ACS2 and LE-ACS4 contain potential *cis* regulatory elements which induce gene expression in the presence of ethylene, or in response to wounding and anaerobiosis. How a single copy gene can

be transcriptionally activated by a diverse group of inducers is poorly understood. Sato and Theologis (1989) proposed that each inducer modifies indirectly or directly the properties of a specific transcription factor. This transcription factor then interacts positively or negatively with a specific DNA domain in the regulatory region of the ACC synthase gene.

Nucleotide sequence analysis of ACC synthase cDNAs from a variety of plants indicates that the deduced amino acid sequences are 72-80% similar and 53-67% identical (Theologis 1992; Li and Mattoo 1994). Comparison of the deduced ACC synthase amino acid sequences demonstrated the presence of seven highly conserved regions including the putative active site (Dong *et al.* 1991). ACC synthase requires pyridoxal phosphate, and most such enzymes have a lysine residue in their active sites. This lysine residue is conserved in all ACC synthases sequenced to date and is the site of pyridoxal phosphate attachment (Theologis 1992).

1.4.2 ACC Oxidase

Unlike ACC synthase, ACC oxidase is constitutively expressed in most vegetative tissues (Yang and Hoffman 1984). However, this enzyme is induced several fold in response to ripening and other stimuli such as wounding and fungal elicitors (Spanu *et al.* 1991; Gomez-Lim *et al.* 1993). ACC oxidase is not as well characterised as ACC synthase, and it is only within the last four years that the gene for ACC oxidase has been cloned and described.

ACC synthase was first discovered by Boller *et al.* (1979), and was then purified from various sources (Kende 1989). The purified protein was used to make antibodies to this enzyme. Genomic DNA libraries were then screened with these antibodies in order to identify clones containing ACC synthase genes (Sato and Theologis 1989). ACC oxidase on the other hand, has remained elusive and difficult to isolate and clone. Although ACC oxidase activity is readily demonstrated *in vivo* by supplying tissues with ACC, the enzyme has been very difficult to characterise due to its instability and inactivation on homogenisation (Vioque and Castellano 1994). It is for this reason that Hamilton and colleagues (1990) used an indirect approach to identify

and clone an ACC oxidase gene. Since ACC oxidase is induced in ripening fruits and wounded plant tissue, Slater *et al.* (1983) screened a fruit ripening related tomato cDNA library with mRNA extracted from wounded leaves. This strategy identified the cDNA clone pTOM13 (Holdsworth 1987). The sequence of pTOM13 did not, however, reveal its function. Expression of pTOM13 antisense DNA in transgenic tomato plants confirmed the role of this gene in the ethylene biosynthetic pathway since the ripening of transgenic tomato fruit was delayed (Hamilton *et al.* 1990). This clone did not code for ACC synthase as its gene product was 35kD compared to the 55kD of ACC synthase (Hamilton *et al.* 1990). Expression of pTOM13 in yeast (Hamilton *et al.* 1991) and in *Xenopus* oocytes (Spanu *et al.* 1991) confirmed that it coded for ACC oxidase as ACC was readily converted to ethylene in both systems.

Confirmation that pTOM13 codes for ACC oxidase has led to the cloning of ACC oxidase genes from other plant species. pTOM13-based probes have identified ACC oxidase cDNA from apples (Dong *et al.* 1992; Wilson *et al.* 1993), avocados (Macgarvey *et al.* 1990), melons (Balague *et al.* 1993), petunias (Tang *et al.* 1993), *Arabidopsis* (Gomez-Lim *et al.* 1993), peas (Peck *et al.* 1993) and kiwifruit (MacDermid and Gardner 1993). Comparison of the various ACC oxidase proteins indicates high homology and suggests common structural features (Gomez-Lim *et al.* 1993).

There is preliminary evidence that ACC oxidase genes may belong to a multigene family in different plant species. Holdsworth *et al.* (1988) showed that tomatoes contain three ACC-oxidase like sequences, whereas avocados contain two (Dong *et al.* 1992), melons have two or more closely related ACC oxidase-like genes (Balague *et al.* 1993), and *Petunia hybrida* has four (Tang *et al.* 1993). Studies on *A. thaliana* ACC oxidase suggest that this plant has only one gene (Gomez-Lim *et al.* 1993). Since ACC oxidase is induced by various stimuli, one functional gene in *A. thaliana* may imply several promoter sequences responding to different signals.

Evidence from the *P. hybrida* ACC oxidase gene family indicates that the 5' flanking sequences exhibit little overall homology between members. The divergence of these sequences could be responsible for conferring unique patterns of expression upon

members of this gene family in response to different stimuli (Tang *et al.* 1993). Members of the ACC oxidase gene family are also differentially expressed in tomato tissues (Holdsworth *et al.* 1988). The ACC oxidase gene homologous to pTOM13 is expressed in leaves and fruit, whereas another ACC oxidase gene (GTOM A) is only expressed in wounded leaves.

ACC oxidase is a soluble cytosolic enzyme (Gray *et al.* 1992) and has an absolute requirement for iron, ascorbate and CO₂ (Smith and John 1993). A stoichiometric analysis of the reaction catalysed by ACC oxidase revealed that ascorbate acts as a co-substrate with ACC producing equimolar amounts of dehydroascorbate and ethylene (Tang *et al.* 1993). CO₂ is necessary for the reactivation of ACC oxidase (Smith *et al.* 1994).

The cloning of ACC oxidase and ACC synthase genes has allowed a more precise analysis of the functions of ethylene during plant development. The most extensively studied of these is the role of ethylene in inducing and coordinating changes in gene expression which occur during ripening of climacteric fruit.

1.5 ETHYLENE PRODUCTION BY SOIL MICROORGANISMS

Ethylene is a common constituent of the soil atmosphere under both aerobic and anaerobic conditions (Considine *et al.* 1977). Soil sterilisation studies have indicated that this ethylene is of microbial origin (Arshad and Frankenberger 1993). Soil ethylene often reaches sufficiently high concentrations to influence plant growth and development. Arshad and Frankenberger (1988) provided direct proof of this theory when they showed that etiolated pea seedlings exhibited the classical triple response to ethylene (reduction in elongation, swelling of the hypocotyl and change to the horizontal direction of growth) when exposed to microbially derived ethylene.

Ethylene-producing bacteria are believed to be ubiquitous in the soil environment (Arshad and Frankenberger 1993). A large number of soil microbes have been tested

for their ethylene forming ability by various researchers. Results of these tests indicate that ethylenogenic microorganisms are overwhelmingly bacteria and fungal moulds, although yeasts and actinomycetes also produce ethylene (Considine *et al.* 1977; Arshad and Frankenberger 1993; Fukuda *et al.* 1993). There are many pathogenic microbes among these ethylene producing microorganisms.

Fukuda *et al.* (1993) have reported that microorganisms and plants synthesise ethylene by different biosynthetic pathways. In microorganisms there are two ethylene biosynthetic pathways, namely the 2-keto-4-methylthiobutyric acid (KMBA) pathway and the 2-oxoglutarate pathway (Figure 1.2). Although L-methionine is the most common precursor of ethylene biosynthesis in microorganisms, there is no evidence that microbial ethylene biosynthesis follows the same pathway as that of higher plants (Arshad and Frankenberger 1993).

In the KMBA pathway, ethylene is produced via KMBA, a transaminated derivative of methionine. This pathway operates in *Escherichia coli* (Ince and Knowles 1986) and probably in most ethylenogenic microbes (Fukuda *et al.* 1993). The conversion of KMBA to ethylene is thought to be through the action of light, as opposed to an enzymic process (Primrose 1977; Fukuda *et al.* 1993).

The 2-oxoglutarate pathway is less common than the KMBA pathway, but has been well characterised in microorganisms such as *Penicillium digitatum* and *Pseudomonas syringae* (Fukuda *et al.* 1993). The purified ethylene forming enzyme from *P. syringae* was found to simultaneously catalyse two reactions, namely the formation of ethylene and succinate from 2-oxoglutarate (Fukuda *et al.* 1993). Comparison of the nucleotide and amino acid sequences of *P. syringae* ethylene forming enzyme with plant ACC oxidases and ripening related enzymes revealed several clusters of invariant residues in the middle section of the sequences (Fukuda *et al.* 1993). Although overall homology was low (15%), functionally significant regions, such as iron binding sites, were conserved.

Although methionine has been found to be the most favourable substrate for ethylene

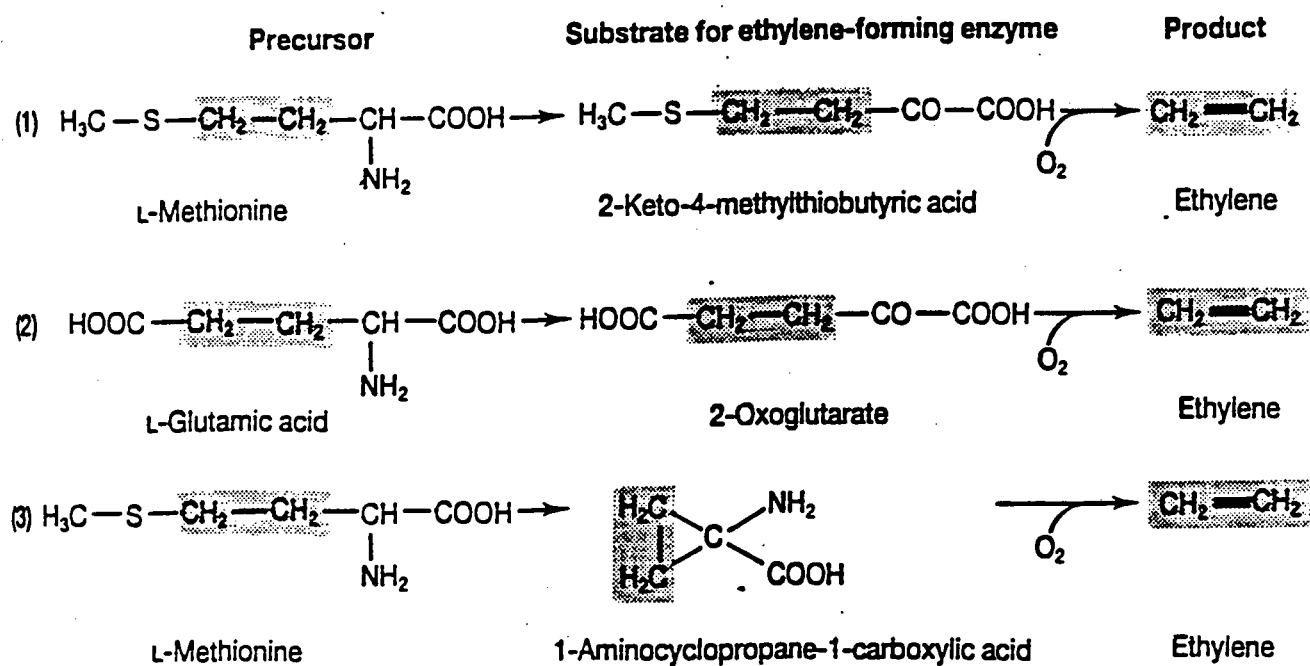


Figure 1.2 Biosynthetic pathways to ethylene in microorganisms and higher plants

(Fukuda *et al.* 1993)

synthesis in microorganisms, a number of microbes are capable of deriving ethylene from various other compounds (Considine *et al.* 1977; Arshad and Frankenberger 1993). These substrates include Krebs cycle intermediates, phenolic acids, organic acids, carbohydrates and amino acid intermediates. However, little is known about how these substrates are converted to ethylene, except that ethylene biosynthesis is affected by varying substrate concentration, carbon and nitrogen content, pH and temperature.

The exact significance and mechanism through which exogenous microbial ethylene influences plant growth is not clear. However, the effects of exogenous hormones on plant growth have been well demonstrated (Arshad and Frankenberger 1993). Microbial hormones have both stimulatory and inhibitory effects depending on the concentration of the hormones, microbial diversity, uptake by plants, and stage of plant development. In turn, the plants' response is governed by the rate of hormone uptake, the active hormone concentration in the rhizosphere, and the plant's own pool of hormones as a result of the exogenous supply. Different crops show different responses to microbial ethylene depending on the plant's genetic make up, the concentration of ethylene, and prevailing environmental factors (Arshad and Frankenberger 1993).

1.6 ALTERATIONS IN FRUIT DURING RIPENING

During the ripening phase of development, enzymes are produced that are involved in the degradation of cell walls, carbohydrates, chlorophyll and many other macromolecules. These enzymes are produced in coordination with enzymes which make the fruit nutritionally and aesthetically desirable.

The molecular basis of fruit ripening has been most widely studied in the tomato (*Lycopersicon esculentum*). This is due to the availability of extensive chromosome maps (Tanksley *et al.* 1989), a variety of ripening mutants (Brady 1987; Picton *et al.* 1993a; Lanahan *et al.* 1994), and a good transformation system (McCormick *et al.* 1986),

combined with the commercial importance of the crop. In 1990, world tomato production exceeded that of bananas, pome fruits and grapes at 6.9×10^7 tonnes (Grierson and Schuch 1993). For this reason most studies examining fruit changes during ripening have been limited to tomato fruits.

1.6.1 Changes in Fruit Colour

Changes in fruit colour during tomato ripening are primarily brought about by the transition of chloroplasts into chromoplasts (Harris and Spurr 1969; Bathgate *et al.* 1985). At an early stage in ripening, chloroplast membranes become disordered, and starch grains and chlorophyll molecules are degraded. As this degradation proceeds, new carotenoid pigments, such as lycopene and its derivative β -carotene, accumulate in the plastid (Bathgate *et al.* 1985; Bird *et al.* 1991). These carotenoid pigments are responsible for giving ripening tomato fruits their characteristic orange to red colour.

Tomato fruit chloroplasts of mature green fruits resemble those of leaf chloroplasts, containing starch grains and photosynthetic membranes arranged into stacks (Harris and Spurr 1969). As fruits begin to ripen there is an unstacking and partial disappearance of the photosynthetic membranes. At this stage chloroplasts lose much of their starch and chlorophyll content. Trebitsch *et al.* (1993) showed that ethylene enhances degreening of the *Citrus* fruit peel through *de novo* synthesis of a chlorophyllase, a chlorophyll degrading enzyme.

Following the breakdown of chloroplast ultrastructure, carotenoids begin to accumulate in fruit cells. Lycopene is found inside the chromoplasts in membrane bound needle shaped crystals. This pigment is deposited on the inner surface of the membrane sacs. As ripening progresses, β -carotene accumulates. Rectangular β -carotene crystals collect in the chromoplast stroma (Harris and Spurr 1969; Bathgate *et al.* 1985). By the time the tomato fruit reaches the ripe red stage, chromoplasts contain large amounts of lycopene, smaller quantities of β -carotene, and reduced levels of stromal thylakoids.

Transformation of chloroplasts to chromoplasts normally occurs simultaneously with other ripening related changes, such as wall softening, but is not essential for ripening (Bathgate *et al.* 1985). Several tomato mutants such as *yellow flesh*, *green flesh* and *tangerine* affect only carotenoid biosynthesis, or chlorophyll degradation, and do not alter other aspects of ripening such as flavour and softening (Frecknall and Tattenden 1984; Gray *et al.* 1992). Furthermore, Bird *et al.* (1991) transformed tomato plants with an antisense gene of the cDNA clone pTOM5. pTOM5 encodes photoene synthase which is involved in lycopene and β -carotene synthesis. Transgenic tomato plants with reduced expression of the pTOM5 gene do not develop red fruit and have low levels of lycopene. Although the yellow fruit have drastically reduced levels of carotenoids, they appear to ripen normally, producing fertile seeds (Bird *et al.* 1991).

1.6.2 Changes in Flavour

Carbohydrate content and composition are important determinants of tomato fruit quality. Carbohydrates affect flavour, which is determined largely by the concentration of sugars and acids, and are a major component of soluble solids, which contribute to tomato processing quality (Klann *et al.* 1993). During ripening the ratio of starch to sugars is decreased due to stored starch being metabolised into sugars within the fruit. In fruits that lack vast starch reserves, sugars are translocated from other parts of the plant (Dali *et al.* 1992). In addition to this increase in sugar content, other flavour components are also synthesised in the ripening fruit. These include organic acids and aromatic compounds, all of which contribute to the final unique balance of sensory components of the ripe fruit.

Bananas accumulate soluble sugars in postharvest conditions from a stored starch reserve, unlike fruits such as tomato which are dependent on concurrent photosynthesis for sugar accumulation. Starch constitutes 20 to 25% of unripe banana fruits, and is almost all converted to soluble sugars during ripening. Initially the predominant sugar is sucrose, but this is converted to glucose and fructose as ripening proceeds (Hubbard *et al.* 1990).

Hubbard *et al.* (1990) demonstrated that starch in bananas is converted to the soluble

sugar sucrose after ethylene treatment. Ethylene enhances the production of the enzyme sucrose phosphate synthase (SPS) which changes starch (stored in the amyloplast) into sucrose. The conversion of starch to sucrose requires ATP, which in harvested bananas is supplied primarily through mitochondrial oxidative phosphorylation. There is evidence that sugar does not accumulate in bananas in the absence of an increase in respiration (Hubbard *et al.* 1990). This suggests a strong link between the ethylene-induced climacteric rise and sugar accumulation.

Sucrose is hydrolysed by the invertase (β -fructosidase) to form its constituents fructose and glucose. An increase in invertase activity in bananas is associated with a decline in sucrose concentration (Hubbard *et al.* 1990). Plant invertases include a variety of forms which can be categorised in terms of solubility, pH optima, isoelectric focusing point, and subcellular localisation (Elliot *et al.* 1993). Acid invertase is located in the vacuole, the site of sucrose accumulation, and neutral invertase is located in the cytosol, the site of sucrose synthesis. The cellular locations of these enzymes suggests that concurrent sucrose synthesis and hydrolysis must occur (Hubbard *et al.* 1990).

Similar results were obtained for muskmelon fruits (Lingle and Dunlap 1987; Hubbard *et al.* 1989). However, unlike bananas, muskmelons accumulate sucrose as opposed to hexose sugars in their final stages of development. Muskmelons lack a significant starch reserve, so carbohydrates are translocated into the fruit during ripening. Furthermore, sucrose accumulation does not occur at the expense of the hexose pool, but rather sucrose is added to the total sugar pool. Stachiose and raffinose are translocated to ripening muskmelon fruits. α -Galactosidase attack upon raffinose sugars releases galactosyl moieties and sucrose. Galactose may be metabolised to sucrose, which in turn may be broken down into hexoses by acid invertase (Hubbard *et al.* 1989).

In tomatoes, the soluble sugar content is a major determinant of fruit quality, both for processing and fresh market uses (Chetelat *et al.* 1993). Tomato paste yield is directly dependent on the solid content, of which soluble sugars are the major component.

It has been shown that tomato species containing high soluble solids content (sucrose) exhibit higher SPS activity and lower invertase activity during development than hexose accumulating *Lycopersicon* sp. (Miron and Schaffer 1991; Dali *et al.* 1992; Klann *et al.* 1993). This is in agreement with results obtained for muskmelons.

Lipoxygenases are a group of enzymes found in plants and animals that catalyse the hydroperoxidation of *cis-cis* pentadiene moieties in polyunsaturated fatty acids. They have been implicated in flavour and odour formation in plants, in plant cell senescence, and in response to pest attack and wounding (Peever and Higgins 1989). Ealing (1994) identified two distinct lipoxygenase activities in fruits which increase sharply during early ripening stages and decrease after the fruit had ripened fully. He speculated that there is a factor present in the early stage of ripening in the fruit pericarp which reacts with lipoxygenases to produce lipid hydroperoxides. These undergo further degradation to produce carbonyl compound like *cis*-3-hexanal, the corresponding *cis*-3-hexanol, which are known to be key flavour components of tomato fruit.

1.6.3 The Softening of Fruits

The softening process is an integral part of ripening in almost all fruits. It has immense commercial importance because the postharvest life of the fruit is to a large extent limited by increasing softness, which brings with it an increase in physical damage during handling and an increase in disease susceptibility. It is for this reason that enzymes involved in fruit softening have been studied more extensively than enzymes involved in other biochemical modifications of the ripening fruit.

Breakdown of stored starch, and later loss of turgor contribute to fruit softening during ripening. However, the most important factor responsible for the softer texture of ripe fruit is believed to be changes to the structure of the celluloses, hemicelluloses and pectins which are the major constituents of the fruit cell wall (Brady 1987). The most important cell wall hydrolysing enzymes are polygalacturonase and cellulase, while other enzymes such as pectinesterase are also thought to be involved. The relative importance of these hydrolases may vary

between species (Gray *et al.* 1992).

Polygalacturonase

Softening is normally accompanied by an increase in the concentration of soluble pectic polysaccharide (Brady 1987). The increase in soluble uronic acid residues is correlated with an increase in the polyuronide hydrolysing enzymes, especially endopolygalacturonase [poly (1,4- α -D-galacturonide) glucanhydrolase] (DellaPenna *et al.* 1986). The tomato polygalacturonase (PG) gene was the first ripening related gene to be identified (Bird *et al.* 1988), and the enzyme is synthesised *de novo* during ripening (Tucker and Grierson 1982). DellaPenna *et al.* (1986) showed that there is a 2000 fold increase in the level of PG mRNA in red ripe tomatoes compared to immature green fruit. PG hydrolyses β 1,4- linkages in the polygalacturonic component of cell walls (Grierson and Schuch 1993).

There are three different isoforms of PG in tomatoes (Tucker *et al.* 1980; Bird *et al.* 1988). These isoforms have been termed PG1, 2a and 2b, and all three forms are believed to be structurally related (Tucker *et al.* 1981). A mixture of PG2a and 2b can be converted *in vitro* to a form resembling PG1 by a heat stable dialysable factor present in tomato fruit extracts (Tucker *et al.* 1981), but the exact mechanism is unclear. PG1 accumulates in the early stages of ripening as the fruit begins to ripen, whereas PG2a is the dominant isoenzyme when the fruit becomes fully ripe (Grierson *et al.* 1986a). All three forms of PG are glycosylated and are probably derived by postranslational modification of a polypeptide encoded by a single PG gene (Smith *et al.* 1990).

The availability of a PG cDNA (DellaPenna *et al.* 1986; Sheehy *et al.* 1987) enabled the gene to be identified from a tomato genomic library (Bird *et al.* 1988). The PG gene is 7.456 kb and includes a 1.4 kb sequence of DNA immediately upstream of the transcription start site which directs ripening specific expression. Southern blots of tomato genomic DNA hybridised to the PG cDNA suggest that there is only one PG gene per haploid genome in tomatoes.

In addition to the established role of PG in polyuronide degradation and fruit softening, it has been suggested that PG dependent polyuronide degradation may release pectic fragments that regulate ethylene synthesis and other components of the ripening process (Tigchelaar *et al.* 1978). Although Campbell and Labovitch (1990) reported that pectic materials generated from tomato fruit cells can induce ethylene biosynthesis in tomato fruit, there are more reports which contradict this finding than favour it (Crookes and Grierson 1983; Grierson and Tucker 1983; Bird *et al.* 1988; Giovannoni *et al.* 1989). Grierson and Tucker (1983) demonstrated that ethylene was important for both lycopene and PG synthesis, and PG activity in tomatoes is only detectable two to three days after the onset of ethylene production.

To elucidate the role of PG during tomato fruit ripening, Giovannoni *et al.* (1989) utilised the pleiotropic genetic mutation *rin* (ripening inhibitor) that blocks many aspects of ripening including the activation of PG gene transcription. The PG gene was introduced into tomato plants, and expression of this gene resulted in the accumulation of active PG enzyme and the degradation of cell wall polyuronides in transgenic *rin* fruit. However this gene had no significant influence on fruit softening, ethylene evolution and colour development of the tomato fruits. Although PG is the most abundant fruit softening enzyme synthesised, it is not responsible for controlling the ripening process and other enzymes along with PG are involved in the process of fruit ripening.

PG activity in the ripening process of fruits has been best investigated in tomatoes. However other fruits such as peaches (Lester *et al.* 1994), avocados (Dopico *et al.* 1993), apples, pears and papayas (Wu *et al.* 1993) also have elevated PG activity during ripening. Apples have also been shown to possess an exo-acting polygalacturonase (Brady 1987).

Other enzymes involved in fruit softening

In tomato fruit PG appears to be the main enzyme produced during fruit softening, while cellulase plays a minor role (Gray *et al.* 1992). The opposite is the case in avocado fruit (Awad and Young 1979). The appearance of cellulase (endo-1,4- β -

glucanase) activity in fruit extracts during ripening correlates with softening of the fruit mesocarp (Christoffersen *et al.* 1984), and cellulase activity, not PG, is closely related to the climacteric rise. This enzyme plays a critical role in cell wall disruption by the cleavage of the linkages between cellulose microfibrils and xyloglucan (Dopico *et al.* 1993). The cellulase mRNA that accumulates during normal ripening is thought to be transcribed from a single ripening related gene (Cass *et al.* 1990).

Pectinesterase is another of the many polysaccharide degrading enzymes that increase in activity during fruit ripening. It functions by removing methyl groups from the pectin components of cell walls. There are at least two pectinesterase isoenzymes expressed in fruits but neither is ripening specific. Although both isoforms are expressed in ripening tomato fruits, the ratios of these change during the ripening process. Pectinesterase 2 is present in large amounts in green fruit before the initiation of ethylene or PG. Similar results were obtained for avocado, orange and banana fruits (Awad and Young 1979). Thus the activity of this enzyme is unlikely to be involved in the initiation of ripening or cell wall degradation. Pectinesterase is believed to have little effect on changes in texture of these ripening fruits, but partial demethylation of pectin is necessary before PG can bring about any significant hydrolysis. Thus pectinesterase may function to prepare the substrate for hydrolysis by PG (Tucker and Grierson 1983).

A number of other glycosidases occur in fruit cell walls (Brady 1987). Some like xylanase in papaya walls (Brady 1987) and xyloglucan endotransglycosylase in kiwifruit (Redgewell and Fry 1993) increase during ripening, suggesting that these enzymes play a role in softening. β -galactosidase plays a role in softening by cleaving galactosidase from the side chains of pectin molecules (Redgewell and Fry 1993).

It can be seen from the above discussion that softening of fruits encompasses a complex series of reactions involving a number of cell wall degrading enzymes. The relative importance in function of these enzymes appears to differ from fruit to fruit. However, it is obvious that all wall degrading enzymes play an interdependent role in ripening, thus contributing to the fruit's postharvest and edible quality.

1.7 MANIPULATING RIPENING WITH ANTISENSE GENES

Every year billions of dollars are lost worldwide due to the overripening of fruits and vegetables during storage and transportation (Sato and Theologis 1989). It is therefore of great importance to the agricultural industry to find a means of controlling the ripening process and preventing fruit spoilage. It has always been a goal to prevent or delay fruit ripening in a reversible manner. Various methods have been employed such as storage of fruits under hypobaric pressure, the use of inhibitors of ethylene (eg. CO₂, Ag²⁺ and aminoethoxyvinyl glycine) or storage at low temperatures (McGlasson 1970). However these approaches are expensive and fail to prevent fruit ripening satisfactorily.

Experiments have shown that it is possible to prevent the effect or expression of existing genes in transgenic plants by introducing a gene constructed to generate antisense RNA (Smith *et al.* 1988; Hamilton *et al.* 1990). This has allowed expression of specific ripening related genes to be curtailed, permitting their identification and assessment of their function in ripening, as well as giving rise to the potential for manipulating fruit ripening (Hamilton *et al.* 1990; Gray *et al.* 1992).

The precise mechanism of antisense RNA action is not known. It has been postulated that antisense genes are specific for a target gene and cause a reduction in the accumulation of normal sense mRNA. It is assumed that transcription of antisense RNA is required for them to be effective (Gray *et al.* 1992). There are two proposed mechanisms of action of antisense mRNA. Firstly it is possible that antisense RNA interferes with the transcription of the target gene. However *in vitro* transcription studies with antisense PG fruit indicate that this does not occur (Sheehy *et al.* 1988). Alternatively antisense RNA might interfere with processing or translation of mRNA. Gray *et al.* (1992) suggested that the translatability of the message is then disrupted by homologous base pairing between the antisense RNA transcript and the target mRNA. The resultant unstable mRNA duplex is then rapidly degraded in either the nucleus or cytosol.

1.7.1 Antisense Inhibition of a Carotenoid Biosynthesis Gene

Expression of an antisense gene to the construct pTOM5, which encodes phytoene synthase, in transgenic plants confirmed that this enzyme is involved in the production of carotenoid pigments during fruit ripening. The total level of carotenoids in ripening fruit from selected transgenic plants with yellow fruit was reduced by more than 97%. In addition the carotenoid lycopene, which is primarily responsible for the red colour of ripening fruits, was reduced to undetectable levels (<0.1%) (Bird *et al.* 1991). [These data indicate that phytoene synthase is crucial to fruit carotenoid biosynthesis]. Although these results are not of great commercial importance as there is no public demand for yellow tomatoes, they help us to understand the complex changes in fruit during ripening. Introduction of this clone into transgenic tomato plants had no effect on delaying the fruit ripening process.

1.7.2 Antisense Inhibition of Fruit Pectinesterase

Ray *et al.* (1988) identified a cDNA for fruit pectinesterase 2. This was used to construct an antisense gene controlled by a CaMV 35S promoter. Pectinesterase antisense RNA inhibited the accumulation of endogenous pectinesterase mRNA in ripening tomato fruit (Tiemann *et al.* 1992; Hall *et al.* 1993). Pectinesterase activity was unaffected in the leaves and roots of transformants, although antisense RNA transcripts were detected in these organs. Residual pectinesterase activity in fruit and other organs was attributed to the expression of one or more other isoenzymes encoded by a gene with insufficient sequence homology to allow inhibition by the product of the antisense gene (Hall *et al.* 1993).

Pectinesterase antisense-expressing tomato plants grew and produced fruit without observable differences from unmodified plants (Hall *et al.* 1993). The transgenic fruit did not appear physically different from the controls at any stage of development or ripening. It was found that pectin remained more heavily esterified (methylated) at all stages of fruit development, thus confirming that pectinesterase has role in pectin de-esterification. However there were no significant differences in softening between ripe control fruit and transgenic tomatoes. Thus pectinesterase 2 does not appear to play a major role in fruit softening.

Tomato fruit expressing pectinesterase antisense RNA have improved processing properties which may be of economic interest to industry (Grierson and Schuch 1993; Hall *et al.* 1993). Low pectinesterase levels lead to an increase in the molecular mass of cell wall pectins. This causes an increase in viscosity of the ripening fruit homogenate, which is a desirable characteristic when selecting for high tomato paste yield (Grierson and Schuch 1993).

1.7.3 Antisense Inhibition of Polygalacturonase (PG)

Smith *et al.* (1988) constructed an antisense PG gene by fusing a 730-bp fragment from the 5' end of PG cDNA in the inverted orientation to a CaMV 35S RNA promoter and the 3' end of the nopaline synthase gene. This 730-bp fragment included a 50-bp untranslated region, and the translation start site. When this construct was introduced into plants there was a striking inhibition of PG activity. Sheehy *et al.* (1987) used a full length PG cDNA in an antisense construct and showed that the rate of transcription of the endogenous PG gene was unaffected in transgenic plants. Rather the steady state level of mRNA that finally accumulated was reduced. Even short (150-bp) segments of the PG cDNA were found to be effective in antisense constructs (Grierson and Schuch 1993).

Smith *et al.* (1990) performed further experiments on transgenic plants with the 730-bp antisense PG construct. These authors found that antisense genes were stably inherited in a Mendelian fashion. Fruit from plants that failed to inherit the transgene had normal levels of PG mRNA and enzyme during ripening (Smith *et al.* 1990). This indicates that the antisense gene did not permanently modify the endogenous PG gene, as it could be recovered by segregation. This rules out the possibility of gene inactivation by homologous recombination (Grierson and Schuch 1993).

Measurement of enzyme activity in a number of transgenic plants demonstrated that PG was down regulated, and reduced activity was clearly an effect of gene dosage (Smith *et al.* 1988; Smith *et al.* 1990). Heterozygous plants containing only one copy of the PG antisense gene showed between 50-95% inhibition of PG activity. However, homozygous plants with two copies of the antisense gene exhibited a 99% reduction

in endogenous PG expression (Smith *et al.* 1988). All three isoforms of PG were inhibited by the antisense construct (Grierson 1992). This adds proof to the idea that all the isoforms are postranslationally modified derivatives of the same gene product.

With the exception of substantially reduced PG activity, the antisense fruit ripened normally. Transgenic plants showed no alteration in ethylene production and lycopene accumulation when compared to controls (Smith *et al.* 1988). Furthermore, there was no deviation from the normal change in activity of invertase which increases during ripening, or in pectinesterase (Smith *et al.* 1990). These results contradict theories which state PG plays a role in coordinating ripening, possibly by stimulating ethylene production.

Despite the dramatic decrease in PG activity, no significant change in the softening of antisense PG transgenics could be detected by compressibility measurements (Smith *et al.* 1988). A major reduction in PG activity would be expected to reduce depolymerisation of cell wall pectin during ripening leading to decreased softening. Results indicated that pectin degradation in the cell walls was inhibited. These findings suggest that although PG is important for pectin degradation, it is not the primary determinant of softening in tomato fruit. Other cell wall degrading enzymes probably make an important contribution to softening.

The role of PG in the softening of tomato fruit is controversial. Kramer *et al.* (1992) showed that transgenic fruit with antisense PG gene constructs from three different fresh market varieties remained firmer after storage when harvested at the mature-green, pink, or red stages of development. On the other hand Smith *et al.* (1988) reported no differences in compression between control and transgenic fruit with reduced PG levels. However field trials have indicated that different genotypes yield different compressibility results for the same antisense PG construct (Kramer *et al.* 1992).

Although transgenic fruits expressing antisense PG RNA soften normally, they still have commercial potential. These fruits are more resistant to cracking, mechanical

damage and secondary fungal infection. These characteristics are probably related to the inhibition of pectin degradation which may cause the cells in the pericarp to be bonded more firmly together (Gray *et al.* 1992).

The PG antisense tomato fruit also have several advantages for processing. Grierson and Schuch (1993) showed that low PG tomato samples are more viscous than controls. Viscosity is influenced largely by the insoluble cell wall polymers. Therefore low PG tomatoes will increase the yield of the paste in the manufacture of tomato paste.

Based on the above observations, Calgene Fresh Inc. have marketed a tomato variety expressing an antisense PG gene. Calgene's Flavr Savr™ tomato stays fresh about 10 days longer than an ordinary tomato and can thus ripen on the vine without rotting (Pfeiffer 1994). This allows the fruit to become redder and riper before being picked for shipment. Fruit allowed to stay on the vine until ripening is at least initiated will develop more flavour than fruit picked green. By contrast, most supermarket tomatoes are picked when still green and then allowed to ripen in the presence of exogenous ethylene. This reddens the tomato fruit but allows no time for the flavour to develop. The Flavr Savr tomato is expected to cost about the same as other premium tomatoes.

The Flavr Savr tomato is the first genetically engineered whole food approved by the Food and Drug Administration (FDA) for sale in supermarkets and grocery stores in the United States. It is believed that this action will open the door to a flood of bioengineered grains, crops, plants, animals and fish. It has been predicted that by the year 2000 over 50 bioengineered foods will be ready for the market (Pfeiffer 1994). However, Calgene is expected to meet with opposition when marketing the Flavr Savr tomato in Britain in 1995 (Young 1994). British critics fear that the kanamycin marker gene present in these transgenic tomatoes may be transferred to intestinal bacteria of mammals. Although extensive research has failed to find proof that marker genes can pass from food to bacteria, antibiotic resistant bacteria are a significant and growing problem in medicine.

1.7.4 Antisense Inhibition of ACC Oxidase

Hamilton *et al.* (1990) introduced an antisense gene to ACC oxidase in tomato plants. These transgenic tomato plants were found to be more resistant to overripening and shrivelling than control plants when stored at room temperature. In these fruit, colour change was initiated at the normal time, although the extent of reddening was reduced. This phenotype was attributed to the inheritance of two antisense genes which inhibited ethylene production by 95%.

Picton *et al.* (1993b) performed further studies on these antisense fruits and showed that the degree of inhibition of ripening was dependent on the stage of development at which the fruit were detached from the plant. Physical detachment of fruit prior to, or at the onset of colour change, exaggerated the slow ripening phenotype. This points to the existence of an additional factor that can affect ripening. This ripening factor is normally provided by the plant, and is either an importable ripening enhancer, or an exportable inhibitor that is translocated from the fruit at the onset of ripening. Alternatively, in attached fruit, there may be a build up of internal ethylene due to the lack of diffusion as the skin is an excellent barrier to ethylene. In unattached fruit almost all of the ethylene diffuses through the stem scar thus delaying the ripening phenotype.

The application of exogenous ethylene only partially restored ripening in detached ACC oxidase antisense-expressing fruits (Picton *et al.* 1993b). Ethylene treatment failed to increase lycopene accumulation of detached transgenic fruit expressing ACC oxidase antisense RNA to the level of the wild type control. Furthermore ethylene treated transgenic fruit demonstrated persistent resistance to overripening and shrivelling (Picton *et al.* 1993b). This suggests that the role of ethylene in fruit ripening may be more complex than a simple switch, and supports the fact that ripening may require a plant associated factor.

1.7.5 Inhibition of Fruit Ripening by Antisense ACC Synthase

Attempts to prevent fruit ripening and spoilage using antisense constructs to ACC oxidase and PG genes were not 100% effective as ethylene production was not

sufficiently decreased to allow effective inhibition of the ripening process. However Oeller *et al.* (1991) reasoned that expression of antisense RNA to ACC synthase, the rate limiting enzyme in the biosynthetic pathway of ethylene, would inhibit fruit ripening in tomato plants. This has been the most successful approach in preventing fruit ripening with antisense technology as it led to severe inhibition of ethylene production.

Oeller *et al.* (1991) expressed antisense RNA to one of the two ACC synthase genes (LE-ACC2) expressed in fruit during ripening (Rottman *et al.* 1991). Expression of antisense RNA derived from LE-ACC2 resulted in an almost complete inhibition of mRNA accumulation of both LE-ACC2 and LE-ACC4. Ethylene production was inhibited by 99.5% and as a result fruits with antisense constructs never ripened. The red colour (of a ripe tomato) resulting from chlorophyll degradation and lycopene biosynthesis was inhibited in antisense-expressing fruits. However ACC oxidase expression remained similar to that observed in control fruits. Transgenic fruits kept in air or on plants eventually developed an orange colour after 90 - 120 days. These fruits never turned red and soft, or developed an aroma.

The antisense phenotype can be reversed by treatment with ethylene or propylene, an analogue of ethylene (Oeller *et al.* 1991). The fruits become fully red and soft after seven days of treatment. Ethylene treatment for one or two days is not sufficient for ripening to occur. This suggests that the ethylene mediated ripening process requires continuous transcription of the necessary genes, and may reflect a short half life of the induced mRNAs or polypeptides. Treated fruits are indistinguishable from naturally ripened fruits with respect to texture, colour, aroma and compressibility (Oeller *et al.* 1991; Theologis 1992).

Tomato plants expressing either ACC synthase and ACC oxidase antisense RNA appear to have normal levels of PG expression in ripening fruit (Oeller *et al.* 1991; Picton *et al.* 1993). These results are in contrast to those of Davies *et al.* (1988) which show a reduction in PG expression in tomato fruit treated with silver ions, a known inhibitor of ethylene perception. This contradiction may be resolved if PG

accumulates in the presence of trace amounts of ethylene, or if silver ions affect pathways implicated in normal PG expression. Picton *et al.* (1993) showed that PG mRNA accumulation is induced in ethylene treated ACC oxidase antisense fruit to a greater level than that observed in control fruit.

Plants expressing antisense ACC oxidase RNA produce ethylene at 5% of normal levels whereas antisense ACC synthase expressing plants synthesise 0.5% of normal ethylene (Hamilton *et al.* 1990; Oeller *et al.* 1991). Oeller *et al.* (1991) suggested that the short half life of the ACC synthase polypeptide is probably an important factor for successful gene inactivation by antisense RNA. Gene transcripts whose encoded polypeptides turn over more rapidly may be inactivated by their antisense RNA more effectively. The less effective inhibition by ACC oxidase antisense genes might be due to the longer half life of the ACC oxidase polypeptide.

Picton *et al.* (1993) disagreed with the above hypothesis. They argued that plants expressing antisense PG genes inhibit expression to less than 1% of the normal level of PG (Smith *et al.* 1988), and PG mRNA and polypeptide are extremely stable (Picton *et al.* 1993). These authors suggested that the difference in levels of ethylene inhibition observed in antisense ACC synthase and ACC oxidase plants is due to the number of independent transgenic plants analysed. Only three antisense ACC oxidase expressing plants were studied (Hamilton *et al.* 1990), whereas thirty four independently transformed ACC synthase antisense-expressing plants were analysed (Oeller *et al.* 1991). Furthermore, Hamilton *et al.* (1990) estimated that plants expressing antisense ACC oxidase RNA producing 5% of normal ethylene, inherited only 2 antisense genes per homozygote. On the other hand, plants expressing antisense ACC synthase constructs produced 0.5% normal levels of ethylene and were reported to have ten copies of the gene per homozygote. Therefore, increasing the antisense gene dosage could increase the severity of the antisense phenotype.

Antisense ACC synthase fruits revealed that at least two signal transduction pathways operate during fruit ripening. The developmentally regulated ethylene independent pathway activates the transcription of genes such as ACC oxidase and chlorophyllase.

On the other hand, the ethylene dependent pathway is responsible for the transcriptional and post transcriptional regulation of genes involved in lycopene and aroma biosynthesis, respiratory metabolism, ACC synthase gene expression and translation of genes. Ethylene appears to have a dual role in senescence. Firstly it activates the transcription of genes whose products are unstable but required for fruit senescence. Secondly it regulates the translation of developmentally regulated mRNAs such as PG (Theologis 1992).

1.8 THE USE OF FRUIT SPECIFIC PROMOTERS

Grierson and Schuch (1993) indicated that CaMV 35S promoter caused antisense PG mRNA to accumulate in several different plant organs, and not just ripening tomato fruit. Most ripening related antisense genes constructed to date are under the control of the CaMV 35S promoter (Hamilton *et al.* 1990; Bird *et al.* 1991; Oeller *et al.* 1991). This situation may not be desirable in the long run as ethylene is involved in plant responses to wounding, pathogen attack, and environmental stress. It is therefore disadvantageous to introduce a gene that controls ripening by preventing ethylene biosynthesis in the whole plant. Such a plant may have weakened responses to wounding and environmental stress. Therefore molecular biologists have undertaken research to identify fruit specific promoters (Bird *et al.* 1988; Deikman and Fischer 1988; Van Haaren and Houck 1991).

Bird *et al.* (1988) characterised the tomato PG promoter. This was found to be a 1.4 kb fragment of DNA immediately upstream of the transcription start site of the PG gene. These authors showed that the PG promoter directed ripening specific expression of the bacterial chloramphenicol acetyl transferase (CAT) gene. This CAT gene was expressed in an organ specific and developmentally regulated manner. No expression occurred in unripe tomatoes, leaves or roots.

The tomato PG promoter is 75% AT rich with putative CAAT and TATA boxes, as well as a 28 bp almost perfect direct repeat (Bird *et al.* 1988). *Cis*-acting DNA sequences in the PG promoter are assumed to contain binding sites for fruit specific

trans-acting proteins required for the formation of a functional transcription complex (Grierson 1992). However the precise location of these hypothetical binding sites have not yet been determined.

Deikman and Fischer (1988) characterised the tomato E8 gene promoter. E8 is a fruit ripening related protein that displays homology to ACC oxidase (Penarrubia *et al.* 1992). E8 is transcriptionally activated at the onset of ripening, coincident with the increase in ethylene biosynthesis. Penarrubia *et al.* (1988) introduced antisense E8 genes into tomato plants. This resulted in an overproduction of ethylene during the ripening of detached tomato fruit. Therefore the E8 protein appears to have a negative effect on ethylene production in fruit.

The sequences required for ethylene responsive and developmentally regulated E8 gene expression in transgenic tomato plants are contained on a 2-kb fragment 5' and 0.5-kb 3' to the gene (Deikman and Fischer 1988). The 2-kb region upstream of the E8 gene has multiple protein recognition sequences. The activation of E8 is correlated with the accumulation of proteins that are able to interact with these recognition sites (Gray *et al.* 1992). Deikman and Fischer (1988) identified a DNA binding factor which interacts with DNA sequences that flank the E8 gene. This DNA binding activity is low in unripe fruit and increases during fruit ripening. This binding factor has also been found to bind to the 5' flanking region of another ethylene responsive gene, the E4 gene, which is coordinately expressed during tomato fruit ripening (Deikman and Fischer 1988). Outside this common binding site, the 5' flanking sequence of E4 and E8 have little homology.

Both the E4 and the E8 gene display temporal and tissue specific modes of expression (Gray *et al.* 1992). Both mRNAs are abundant in ripening fruit, but are rarely found in unripe fruits, leaves, stems and roots. As a result both promoters have been used to drive fruit specific expression of particular genes (Giovanni *et al.* 1989; Kellog *et al.* 1994). However, when whole tomato plants are exposed to ethylene the E4 gene is expressed in many plant organs, whereas the E8 gene is only expressed in fruit (Gray *et al.* 1992). The E8 promoter is therefore better for fruit specific expression of genes.

Van Haaren and Houck (1991) obtained fruit specific expression of GUS when this gene was flanked by the 5' and 3' regions of the tomato 2A11 gene. Martineau *et al.* (1994) obtained similar results when expressing an *Agrobacterium tumefaciens* isopentenyl transferase gene in tomato. The 3' region of the 2A11 gene plays a minor role in fruit specific expression and can be deleted (Van Haaren and Houck 1991). The 4-kb 5' flanking region of this gene contains regulatory elements extremely far upstream from the transcriptional start of the gene (Van Haaren and Houck (1991).

Compared to other developmentally regulated promoters, and particularly fruit specific promoters, the 2A11 gene is tightly regulated and has regulatory elements extremely far upstream from the transcriptional start of the gene (Gray *et al.* 1992). Although the 2A11 protein is only detected in fruit, it is not ripening specific. 2A11 mRNA first appears at anthesis and reaches a maximum level in mature fruit.

The 4-kb 5' flanking region of the 2A11 gene has several regulatory elements (Van Haaren and Houck 1988; Gray *et al.* 1992). There are seven positive regulatory elements; four are fruit specific and three are general. In addition three negative regulatory elements are present. DNA binding studies indicated that a number of proteins interact with these regulatory sequences (Gray *et al.* 1992). However, a sequence comparison of the 5' region of the 2A11 gene with the upstream regions of the PG, E4 and E8 genes did not reveal the presence of any conserved sequences (Van Haaren and Houck 1991).

So far only tomato fruit specific promoters have been characterised. Studies now need to be undertaken to identify more ripening related promoters in tomatoes, as well as other climacteric fruits.

1.9 OTHER MOLECULAR METHODS OF CONTROLLING RIPENING

As outlined in the previous section, antisense genes to key enzymes involved in fruit ripening are effective in controlling the ripening process. However, although these

antisense constructs are effective in the species of origin and closely related species, different antisense constructs have to be made for distantly related species as there is not enough homology between the same genes of different plants. For this reason researchers have set out to identify genes that are able to control ripening in a variety of plant species. Klee *et al.* (1991) and Good *et al.* (1994) have been successful in this task. They identified genes from bacteria and bacteriophage respectively that have the potential to inhibit ethylene synthesis in a number of different plant species.

1.9.1 Control of Ripening with s-adenosylmethionine hydrolase

S-adenosylmethionine (SAM) is the immediate precursor of ACC, and the penultimate precursor to ethylene. Kellog *et al.* (1994) have isolated an s-adenosylmethionine hydrolase (SAMase) gene from T3 bacteriophage. This enzyme catalyses the conversion of SAM to methylthioadenosine (MTA) and homoserine, which reenter the methionine cycling pathway (see Figure 1.1). Therefore, SAM is not available for the synthesis of ACC and ethylene biosynthesis is inhibited.

When the SAMase gene was placed under the control of the E4 promoter, ethylene synthesis was reduced by 20-40% in transgenic tomato plants (Kellog *et al.* 1994). This led to delayed fruit ripening and an extended shelf life of fruit. Field trials showed that transgenic plants displayed normal growth and that genetic engineering had no deleterious effect on field production of tomato fruit.

Good *et al.* (1994) obtained similar results when an SAMase gene fused to the E8 promoter was introduced into tomato plants. Field trials indicated that transgenic tomatoes displayed increased firmness, and this was maintained for several weeks. Vitamin A and C and glycoalkaloid content remained the same as the controls. SAMase only constituted between 0.05% and 1% of the total soluble protein of fruit, and will have little effect on tomato taste and edibility.

1.9.2 Control of Ripening with Bacterial ACC Deaminase

Klee *et al.* (1991) used a different approach to control the ripening of climacteric fruits.

These authors introduced a bacterial ACC deaminase gene into tomato plants. The fruits from these plants exhibited significant delays in ripening and the mature fruits remained firm for at least 6 weeks longer than the non-transgenic control fruit.

ACC deaminase inhibits fruit ripening by metabolising ACC, the immediate precursor of ethylene. ACC deaminase utilises a pyridoxal 5' phosphate as a cofactor in catalysing the cleavage of ACC to α -ketobutyrate and ammonia (Honma and Shimomura 1978). Both of these reaction by-products are found naturally in plants. Furthermore α -ketobutyrate is the substrate for the enzyme acetolactate synthase, and is converted to branched chain amino acids in plants (Klee *et al.* 1991).

The rate limiting step in ethylene biosynthesis in plants is the conversion of SAM to ACC by ACC synthase. The reverse of this reaction can be viewed as analogous to the reaction catalysed by ACC deaminase. Sheehy *et al.* (1991) compared the amino acid sequence of an ACC deaminase from *Pseudomonas* sp. strain ACP with the deduced amino acid sequence for a tomato ACC synthase. This revealed two regions of greater than 70% similarity, one of which coincided with the active site of ACC synthase. However, the significance of this homology is unknown.

A number of ACC deaminase producing microorganisms have been identified. Honma and Shimomura (1978) isolated a *Pseudomonas* sp. and a yeast, *Hansenula saturnus*, that were able to degrade ACC. The *Pseudomonas* sp. ACC deaminase has a molecular weight of 104 kD and has a much higher specific activity than the yeast ACC deaminase which has a molecular weight of 69 kD. The reason that ACC degrading microorganisms are readily isolated from the soil is probably because of the abundance of ACC in decaying plant material.

Klee *et al.* (1991) isolated a *Pseudomonas* sp. ACC deaminase gene and introduced it into tomato plants. Analysis of transgenic fruit showed that there is a correlation between the degree of ethylene inhibition and the delay in the progression of ripening. Visual observation of transgenic fruit indicated a significant reduction in softening, with fruit remaining firm for longer than five months. On the other hand,

control fruit stored for the same period of time were almost completely desiccated (Klee *et al.* 1991).

Interestingly, biochemical analyses of transgenic fruit indicated no significant differences from controls in levels of ACC oxidase or PG (Klee 1993). However it is impossible at this time to rule out the crucial role of ethylene in the induction of ACC oxidase or PG. Since ACC deaminase producing plants reduce ethylene production by 90-97% , residual levels of ethylene may be all that is necessary to trigger PG and ACC oxidase synthesis. However the lack of difference of PG between the firmer transgenic fruit and controls indicates that another unidentified activity must be critical for the softening that ultimately leads to spoilage (Klee 1993).

Klee (1993) found physiological differences in transgenic fruit ripened on and off the vine. ACC deaminase producing fruit ripened significantly slower than control fruit when removed from the vine. In contrast, fruit that remained attached to the plants ripened more rapidly with little delay relative to the control. Ethylene determinations of attached fruit revealed that there was significantly more internal ethylene in attached than detached fruit. The higher ethylene content accounts for the faster ripening on the vine. Likewise, these observations suggest that the plant could synthesise factors that are translocated to fruit and are promotive to ripening. Such a conclusion was also drawn by Picton *et al.* (1993) from work done on attached and detached antisense ACC oxidase fruit.

Transgenic plant lines producing ACC deaminase show that there is a correlation between the amount of enzyme produced and the degree of ethylene inhibition. One line with an ethylene reduction of 97% contained approximately 0.5% of the soluble protein in ACC deaminase. However, a line that reduced ethylene synthesis by only 78% contained only 0.05% ACC deaminase in its soluble protein extract (Klee 1993).

Degradation of ACC inhibits ethylene synthesis but does not interfere with the fruit's ability to detect and respond to the presence of exogenous ethylene. Transgenic fruit exposed to exogenous ethylene ripen normally (Klee 1993). These factors, together

with the fact that transgenic ACC deaminase plants remain firmer than controls, indicate that expression of ACC deaminase should result in a significantly extended shelf life for tomatoes and other climacteric fruits.

The findings of Klee *et al.* (1991) and Klee (1993) demonstrated that a bacterial ACC deaminase gene is effective in inhibiting ethylene synthesis and delaying tomato fruit ripening. This will allow for an extended storage and shelf life for transgenic fruit.

Inhibition of ethylene synthesis by ACC deaminase genes compares favourably with inhibition by antisense ACC synthase and ACC oxidase genes. Transgenic ACC deaminase tomatoes inhibit ethylene synthesis by 97% compared with 99% and 95% for ACC synthase and ACC oxidase respectively. Furthermore inhibition of ethylene synthesis by the introduction of an ACC deaminase gene has the advantage of being effective in a number of plant species. Antisense constructs are specific for one plant species only.

Based on the above findings, the objective of this study was to clone an ACC deaminase gene, and to make a suitable construct to express this gene in a number of different plant species to delay fruit ripening.

CHAPTER II

Screening Microorganisms for ACC Deaminase Activity

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2.1 SUMMARY

Soil microorganisms were screened for ACC deaminase activity on minimal medium with ACC as the sole nitrogen source. Of the eighty-three microorganisms displaying ACC degrading ability, nine were chosen for further analysis.

Enzyme assays showed that *Pseudomonas* species had the highest ACC deaminase activity. This activity was higher than that previously reported for *Pseudomonas* sp. strain ACP (Honma and Shimomura 1978). Yeast ACC deaminase activity was very low although growth was as vigorous as Pseudomonads on ACC minimal media plates.

PCR and Southern blot analysis indicated that only one of the selected Pseudomonads, *Pseudomonas* sp. strain 17, had an ACC deaminase gene homologous to those sequenced from *Pseudomonas* sp. strain 65G (Klee *et al.* 1991) and *Pseudomonas* sp. strain ACP (Honma and Shimomura 1978). All other Pseudomonads and yeasts appeared to have novel ACC deaminase genes.

2.2 INTRODUCTION

A mechanism of preventing ethylene synthesis would involve irreversible degradation of the precursor of ethylene, ACC, to an inactive compound. Current knowledge of ethylene biosynthesis in plants indicates that ACC is the immediate metabolic precursor of ethylene under physiological conditions (Yang and Hoffman 1984). Besides ethylene biosynthesis, only two metabolic reactions of ACC are known (Klee *et al.* 1991). The first involves the degradation of ACC to α -ketobutyrate and ammonia by the bacterial enzyme ACC deaminase. The second takes place in plant cells where ACC is malonylated to yield malonyl-ACC (McKeon and Yang 1987).

A number of ACC degrading microorganisms have been identified. Honma and Shimomura (1978) screened microorganisms isolated from soil on media containing ACC as the sole nitrogen source. These authors identified several microorganisms utilising ACC as a nitrogen source, among which bacteria and yeasts were included. Klee *et al.* (1991) screened 600 microorganisms isolated from four different continents for ACC deaminase activity. Three organisms were isolated from this screening, two of which were further identified as being *Pseudomonas* species. Microorganisms capable of degrading ACC can be readily isolated from the soil probably because of the abundance of ACC and its metabolite malonyl-ACC in decaying plant material.

Our aim was to isolate ACC degrading soil organisms and identify the microbe with the highest ACC deaminase activity. It was decided to screen these microorganisms to see how many of them had ACC deaminase genes which differed from those isolated by Sheehy *et al.* (1991) and Klee *et al.* (1991).

2.3 MATERIALS AND METHODS

Standard materials and referenced methods are recorded in the Appendix.

2.3.1 Microorganisms and culture conditions

103 potential ACC degrading microorganisms were obtained from the Agricultural Research Council's (ARC) Institute for Fruit Technology (Infruitec). These microorganisms were isolated from soil samples taken from various research stations throughout South Africa (I. Roos, personal communication). The ACC degrading strains *Pseudomonas* sp. strain ACP and the yeast, *Hansenula saturnus* var. *saturnus*, were acquired from Honma and Shimomura (1978). All cultures were grown and maintained on Luria-Bertani (LB) agar plates and stored on LB agar slants.

2.3.2 Microbial Screen

The microorganisms were screened for ACC degrading ability. The screen was designed to select for organisms that would grow on minimal medium containing 3 mM ACC as the sole nitrogen source.

Samples were grown on medium consisting of DF salts (Dworkin and Foster 1958) minus nitrogen, and 15% Bacteriological No. 1 agar (Oxoid). The medium was supplemented with 3 mM ACC, and 0.2% of glucose, gluconic acid and citric acid. Samples were left to grow at 30°C for 2 to 4 days. Cultures were also grown on control plates consisting of the above medium without 3 mM ACC or any other nitrogen source.

2.3.3 Identification of ACC degrading microorganisms

Nine ACC degrading microorganisms were chosen for further analysis. Dr I. Roos from Infruitec performed standard identification procedures to determine the genera of these microorganisms. Identification of the two microorganisms with the highest ACC deaminase activities was performed using an Api 20 NE kit (Bio Merieux SA) as outlined by the manufacturer.

2.3.4 ACC deaminase assays

The ACC deaminase activity of ACC degrading microorganisms was measured by following the production of α -ketobutyric acid. This was measured with 2,4-dinitrophenylhydrazine derivitisation as described by Honma and Shimomura (1978).

Pseudomonas species were first cultured by shaking them at 30°C for 40 h in a medium composed of 2% glucose, 0.5% peptone and 0.3% dried yeast extract (YPD medium). Cells harvested by centrifugation were washed with 0.1 M potassium phosphate, pH 7.4, and further incubated in a second medium containing 1% sucrose and 0.15 mM ACC in an organic salt solution. This salt solution consisted of 0.1% K_2HPO_4 , 0.1% KH_2PO_4 , 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% $CaCl_2 \cdot 2H_2O$ and 0.013% $FeSO_4 \cdot 7H_2O$. Cells were incubated in this second medium at 30°C for 4.5 h before they were collected by centrifugation, and resuspended in 5 ml 0.1 M potassium phosphate buffer, pH 7.4. Cells were ruptured by a high pressure extrusion method with a French Press (French and Milner 1955). Crude cell extracts were centrifuged to remove any cell debris present in the sample.

Yeast cells were collected by the same procedure as above. The first culture was grown in a medium composed of 3% sucrose, 2% KNO_3 and the inorganic salt solution. The second incubation medium was the same as the first except that ACC replaced KNO_3 as the sole nitrogen source.

Aliquots (0.1 ml) of crude cell free extracts were incubated with 100 mM ACC prepared in 0.1 M Tris-Cl, pH 8.5. Samples were left for 2 h at 30°C before stopping the reaction with 1.8 ml 0.56 N HCl. Dinitrophenylhydrazine-2 N HCl (0.3 ml of a 0.1% solution) was added, and the samples were incubated at 30°C for 15 min. After the addition of 2 ml 2 N NaOH, the A_{540} of the solutions were read on a Beckman Du^R-64 spectrophotometer. The α -ketobutyrate concentration was determined from a standard curve of absorbance at 540 nm versus α -ketobutyrate concentration.

2.3.5 Protein concentration determination

Protein concentrations of the crude cell free extracts were determined using the

Bradford reagent (BioRad Laboratories), with bovine serum albumin as a standard.

2.3.6 PCR amplification

PCR amplification reactions contained 0.125 μ M of each primer, 10% DMSO, 200 μ M of each nucleotide, 10 mM MgCl₂ and crude template DNA in the form of bacterial cells. Promega *Taq* DNA polymerase and buffer were used in all reactions.

Reaction mixtures were subjected to 30 cycles of amplification using a denaturation step of 93°C (60 sec), an annealing step of 44°C (30 sec), and an extension step of 72°C (60 sec). A final extension reaction at 72°C for 5 min was included. DNA amplification cycles were controlled by a custom made programmeable thermocycler (JDI Model 8012). After amplification, samples were electrophoresed through a 1% agarose gel, stained with EtBr and viewed over a 264 nm light source.

2.3.7 Southern blot analysis of ACC degrading microorganisms

A partial 817-bp ACC deaminase gene probe from *Pseudomonas* sp. strain ACP was prepared using PCR amplification. However, nucleotides were replaced with an equal concentration of 10X digoxigenin-II-dUTP (DIG) DNA labelling mix (Boehringer Mannheim). After amplification, the sample was run through a 0.8% agarose gel in TAE buffer. The band corresponding to the correct size was cut out of the gel and DNA was eluted using the GeneClean^R procedure as stated by the manufacturer.

Pseudomonas sp. genomic DNA was prepared according to the method of Ausubel *et al.* (1989). Yeast genomic DNA was isolated as described by Sanglard (1983). Approximately 5 μ g DNA was digested for 3 h at 37°C with 5 units of *PvuII* and run on a 1% agarose gel in TBE. Genomic DNA was depurinated in 0.25 N HCl for 10 min before being rinsed in distilled water, and capillary blotted with 0.4 M NaOH onto Hybond N⁺ (Amersham). The blot was probed with a DIG-labelled probe (Boehringer Mannheim). Hybridisation signals were detected via chemiluminescence using the AMPPD^R substrate (Boehringer Mannheim).

2.4 RESULTS AND DISCUSSION

2.4.1 Identification of ACC degrading microorganisms

Eighty-one of the 103 microorganisms screened displayed ACC degrading ability. These included bacteria, yeasts and two filamentous fungi. Growth of these microorganisms on minimal medium plates containing ACC as the sole nitrogen source varied from pinhead size colonies to a bacterial lawn after four days growth. None of these ACC degrading microorganisms grew on control plates lacking a nitrogen source. This ruled out the possibility that these organisms could fix nitrogen as opposed to degrading ACC.

Nine microorganisms showing the most vigorous growth on ACC minimal medium plates, were selected for further identification. Tests indicated that seven of these microorganisms were fluorescent *Pseudomonad* species as bacterial colonies fluoresced under UV light when plated on King's B medium (Stolp and Gadkari 1981). The remaining two ACC degrading microorganisms were yeast species.

Pseudomonads appear to be the most abundant microorganisms with ACC deaminase activity. Klee *et al.* (1991) screened 600 microorganisms for ACC degrading enzymes and identified three microbes from this screening, two of which were *Pseudomonas* species. Honma and Shimomura (1978) also identified a *Pseudomonas* with ACC deaminase activity. *Pseudomonads* are frequently isolated from acid media rich in sugar, such as flowers, decaying or "rotten fruits", and various other plant materials (Palleroni 1991). Because ACC and its derivative malonyl-ACC are abundant in plant material, it would be advantageous for these saprophytic *Pseudomonads* to utilise ACC as a nitrogen source.

2.4.2 ACC deaminase activity in soil microorganisms

ACC deaminase degrades ACC to form α -ketobutyrate and ammonia. Cell free extracts from the selected *Pseudomonads* and yeasts were prepared and assayed for ACC deaminase activity by measuring the production of α -ketobutyrate with 2,4-dinitrophenylhydrazine as a colorimetric indicator (Honma and Shimomura 1978).

One unit of ACC deaminase activity is defined as the activity forming 1 μ mol of α -ketobutyrate per minute. Specific activity of an enzyme is the number of enzyme units per milligram of protein present in the assay reaction.

Results (Figure 2.1) indicate that the ACC degrading *Pseudomonads* display high ACC deaminase activity. The specific activity of ACC deaminase harboured by these *Pseudomonads*, as well as that of the control *Pseudomonas* sp. strain ACP, was five to six times higher than the specific activity determined by Honma and Shimomura (1978) for *Pseudomonas* sp. strain ACP crude cell extract. These authors obtained a specific activity of 0.206 U/mg protein for this organism. The fluorescent *Pseudomonad*, TL6, which does not grow on ACC minimal medium, showed no ACC deaminase activity.

Sheehy *et al.* (1991) defined one unit of ACC deaminase activity as a change in A_{540} of 0.001 per minute due to the conversion of ACC to α -ketobutyric acid. When the enzyme activities of selected *Pseudomonads* were recorded according to this unit definition, specific activities ranged from 55 U/mg to 111 U/mg protein (Figure 2.2). The specific activity of *Pseudomonas* sp. strain ACP was 90 U/mg of protein. This was approximately three times higher than the specific activity of 29.5 U/mg protein obtained by Sheehy *et al.* (1991).

Possible reasons for discrepancies between results in this study and those of other researchers could include differences in growth conditions [Sheehy *et al.* (1991) grew the *Pseudomonas* cells in a different induction medium] and possible differences in assay technique. In this study the *Pseudomonad* cells were grown in the first culture medium (YPD) for 40 h as outlined by Honma and Shimomura (1978). However, the bacteria were then incubated in the ACC deaminase inducing medium for 4.5 h. Honma and Shimomura (1978) did not state how long the *Pseudomonas* sp. strain ACP was left in this medium.

Yeast strains 6 and 14 showed very little ACC deaminase activity (Figure 2.1). This

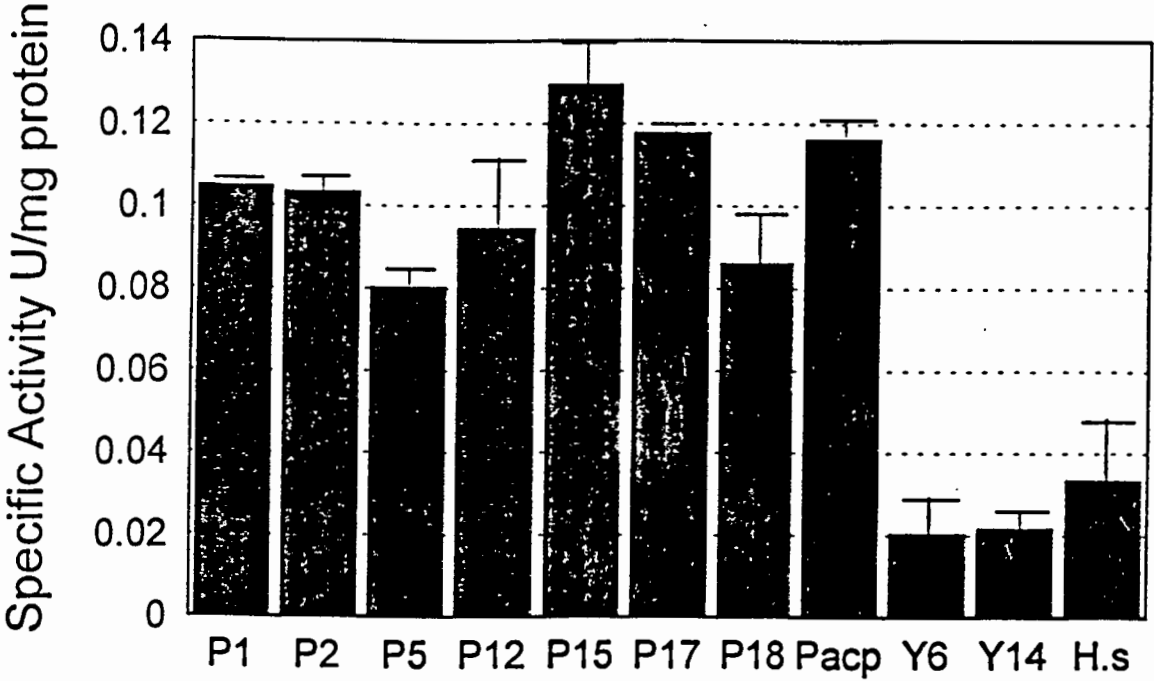


Fig. 2.1 Specific activities of ACC deaminase from ACC degrading soil microorganisms.
One unit of the enzyme indicates the activity forming 1 μ mol of α -ketobutyrate (Honma and Shimomura 1978). Bars represent standard deviations.
P = *Pseudomonas*; Y=yeast.

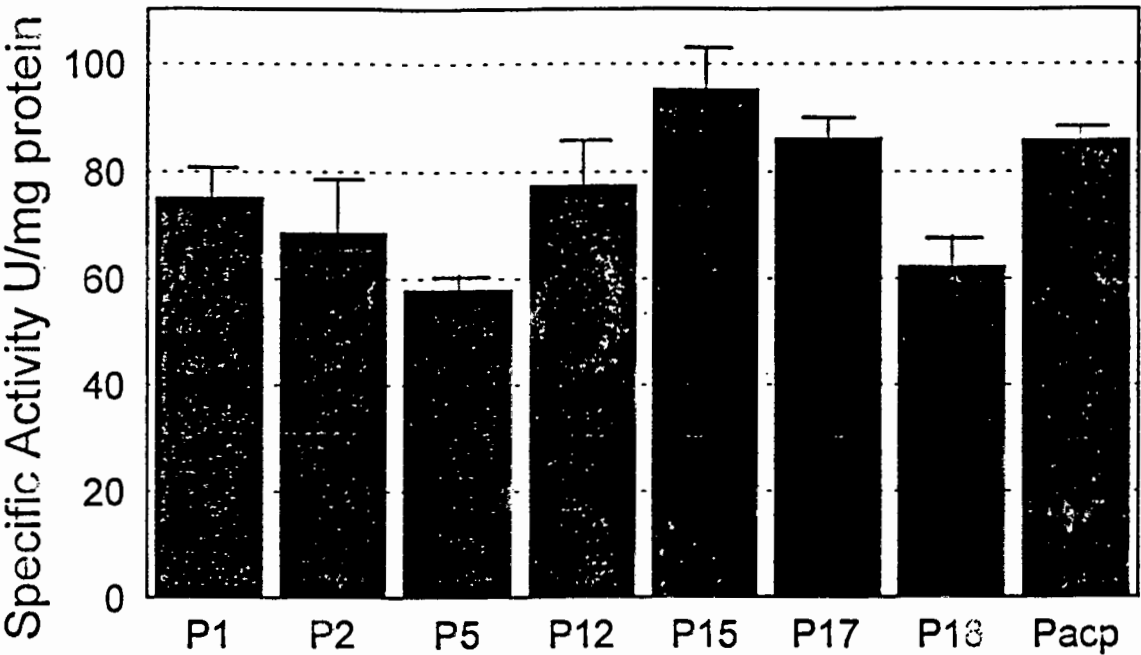


Fig. 2.2 Specific activities of ACC deaminase from ACC degrading soil microorganisms.
One unit of ACC deaminase activity is defined as a change in A_{540} of 0.001 per minute (Sheehy *et al.* 1991). Bars represent standard deviations. P = *Pseudomonas*.

result was surprising as the yeast cells appeared to grow as well as the *Pseudomonads* on minimal medium containing ACC as the sole nitrogen source.

ACC deaminase from the control yeast *Hansenula saturnus* var. *saturnus* had a specific activity of 0.0477 U/mg protein. Surprisingly this activity was approximately nine times higher than the value of 0.00504 U/mg protein obtained by Honma and Shimomura (1978) for the same organism.

Extraction techniques were varied for yeast strains 6 and 14 in an attempt to improve ACC deaminase specific activity. These changes included extracting the enzyme at 4°C, and storing the cell free extract in 0.25 M sucrose which often stabilises enzymes (G. Lindsay, personal communication). However, neither of these changes had an effect on ACC deaminase activity. A possible reason for low ACC deaminase activity could be in the French Pressing procedure itself. Although all samples were French Pressed twice, ACC deaminase as well as total protein concentrations were very low for yeast strains. Honma and Shimomura (1978) disrupted yeast cells by a high pressure extrusion method in a cell disrupter (Stansted Fluid Power Ltd) as opposed to a French press.

2.4.3 Screening for novel ACC deaminase genes

ACC deaminase nucleotide sequences from *Pseudomonas* sp. strain 65G (Klee *et al.* 1991) and *Pseudomonas* sp. strain ACP (Sheehy *et al.* 1991) were aligned with the aid of programmes from the Genetics Computer Group (GCG, University of Wisconsin) package version 8.0 (Devereux *et al.* 1984) run on a DEC/Vax 6000-330 mainframe computer (Figure 2.3). Primers were designed, following the guidelines of Innis and Gelfand (1990), to the most conserved regions that allowed maximum amplification of the gene. The sequences of forward and reverse primers are shown in Figure 2.4. Forward primers are identical to the sequence between regions 100 and 123 of the ACC deaminase genes of *Pseudomonas* sp. strains 65G and ACP. Reverse primers were complementary to the sequence 893 to 914 of the gene from the same organisms at the 3' end. All four primers were used in each PCR reaction to amplify an 817 bp fragment of the ACC deaminase genes. This fragment contains the predicted co-factor binding site of pyridoxal 5'-phosphate at position 151-153 (amino acid 51) of

```

      10      20      30      40      50      60
Pse65g ATGAATCTGAATCGTTTGAACGTTATCCATTGACCTTCGGTCTCTCCATCACGCC
Pseacp ATGAACCTGCAACGATTCCCTCGTTACCGCTGACTTTCGGGCGGACGCCAATCCAACCG

      70      80      90      100     110     120
Pse65g TTGAAGCGCCTCAGTCAACATCTGGGGGCAAGGTCGAGCTGTATGCCAAACGTGAAGAC
Pseacp CTAGCGGCTCTGAGCAAGCACCTCGGCGGCAAGTGCATCTGTATGCCAAACGCGAAGAC

      130     140     150     160     170     180
Pse65g TGCAACAGTGGCCTGGCCTTGGTGGGAACAAGACGCGCAAGCTCGAATACCTCATT CC
Pseacp TGCAACAGCGGCTGGCCTTGGTGGCAACAAGACGCAAGCTCGAATATCTGTATC CT
      ▶

      190     200     210     220     230     240
Pse65g GAAGCGATCGAGCAAGGCTGCGATACGCTGGTTTCCATCGGGCGGCATCCAGTCCGAAC AG
Pseacp GAAGCGCTTGCTCAGGTTGCGACACGCTCGTGTGATCGGCGGCATTCTAGTCCGAAC AG

      250     260     270     280     290     300
Pse65g ACCCGTCAGGTCGCTGCGCTCGCTGCCCACTTGGGCATGAAGTGCCTGTTGGTGCAG AA
Pseacp ACGCGCCAGGTGGCGGCGTGGCGGCTCATCTGGGCATGAAGTGCCTGCTGGTGCAGGAG

      310     320     330     340     350     360
Pse65g AACTGGGTGAACTATTCCGACGCGGTGTATGACCGCTAGGCAACATCGAGATGTCGCGG
Pseacp AACTGGGTCAACTATTCCGACGCGGTGTATGACCGCTAGGCAACATCCAGATGTCGCGG

      370     380     390     400     410     420
Pse65g ATCATGGGCGCTGATGTGCGGCTTGACCGCGCTGGCTTCGATATTGGCATTCCGCCAAGT
Pseacp ATTCTCGGCGCGATGTTCCGCTCGTGGCGGCGCTTCGACATCGGTTTTCGAGGAGC

      430     440     450     460     470     480
Pse65g TGGGAAAAGGCCATGAGCGATGTCGTGAACAGGTTGGCAACCGTTTCCGATTCCAGCG
Pseacp TGGGAGGATGCGCTGGAAGCGTGGCGGCGCGCGGCAAGCGTATGCGGATTCGCGCA

      490     500     510     520     530     540
Pse65g GGTGTCTCCGAGCATCCCTATGGCGGCGCTCGGTTTCTGTCGGCTTTCGCGAAGAGGTCCGG
Pseacp GGCTGCTCGGATCACCCGCTCGGCGGCGCTGGGTTTCTGTCGGCTTCGCGGAGGAGGTCCGG

      550     560     570     580     590     600
Pse65g CAGCAGGAAAAGGAAGTGGGCTTCAAGTTTGACTACATCGTGGTCTGCTCGGTGACCGGC
Pseacp GCGCAGGAAGCCGAATTGGGCTTCAAATTCGACTATGTCGTGCTGTTCCGTGACCGGC

      610     620     630     640     650     660
Pse65g AGTACGACGCGCGGCTGTTGTTGTTTCGCGGCTGACGGTCTGTCGAAGAATGTGATT
Pseacp AGCAGCGACGCGCGGCTGTTGTTGTTTCGCGGCTGACGGCGCGCGGATCGCGTGATC

      670     680     690     700     710     720
Pse65g GGTATCGATGCTTCGGCCAAGCCGGAACAGACCAAGGCACAGATCCTGCGCATCGCCCGA
Pseacp GCGCTCGACGCTTCGGCCAACCCGCGAGCGCGGAGCAGATCACCCGCATCGCGAGA

      730     740     750     760     770     780
Pse65g CACACCGCTGAGTTGGTGGAGTTGGGGCGCGAGATTACGGAAGAGGACGTGGTGTCTCGAT
Pseacp CAGACCGCGGAGAAATCGGCCTGGAGCGCGATATCATGCGGGCCGACGTGGTGTCTCGAC

      790     800     810     820     830     840
Pse65g ACGCGTTTTGCCTACCCGGAATATGGCTTGCCCAACGAAGGCACATTGGAAGCCATCCGA
Pseacp GAGCGCTTCGCGGCTCCGGAATACGGATTGCGGAACGAAGGCACGCTGGAAGCGATCCGC

      850     860     870     880     890     900
Pse65g CTGTGCGGCAGCCTTGAAGGCGTGTGACAGACCCGGTATATGAAGGTAATCGATGCAC
Pseacp TTGTGCGCGCGCACGAGGGCATGCTGACCGATCCGCTCTACGAAGGCAATCGATGCAC
      ◀

      910     920     930     940     950     960
Pse65g GGCATGATTGAAATGGTCCGTCGTGGTGAATTCGCCGAAGGTTCCAAAGTGCTTTACGCA
Pseacp GGCATGATCGAAATGGTCCGCAACGGCGAATTCGCCGAAGGCTCGCGCGTGTGTATGCG

      970     980     990     1000    1010
Pse65g CACTTGGGTGGGGCGCGCGCTGAACGCTACAGCTTCCTGTTTCGTAACGGCTAA
Pseacp CACCTCGGCGGGTGCGGCGTTGAACGGCTACAGCTTTATCTTCGAGACGGCTGA

```

Fig. 2.3 Alignment of the ACC deaminase gene sequences from *Pseudomonas* sp. strain 65G and *Pseudomonas* sp. strain ACP.

PCR primers were constructed to the most conserved regions that allowed maximum amplification of the ACC deaminase gene. Arrows represent the binding sites of forward and reverse primers. The sequence coding for the predicted cofactor-binding lysine is boldly underlined.

Pseudomonas sp. strains 65G and ACP.

- A. 5'-CTGTATGCGAAACGCGAACACTGC-3'
- B. 5'-CTGTATGCCAAACGTGAAGACTGC-3'
- C. 5'-ACCATTTCATCATGCCGTGCATCG-3'
- D. 5'-ACCATTTCGATCATGCCGTGCATCG-3'

Fig. 2.4 Oligonucleotide primers used for PCR reactions.

A and B are forward primers and differ at positions 9 and 15. Primers C and D are reverse primers and differ at position 9.

PCR amplification from crude DNA extracts of the nine selected microorganisms showed that only *Pseudomonas* sp. strain 17 had an ACC deaminase gene similar to that of *Pseudomonas* sp. strain 65G and *Pseudomonas* sp. strain ACP. Amplification of *Pseudomonas* sp. strain 17 DNA with ACC deaminase gene specific primers produced an 817-bp PCR product that was the same size as that of the control bacterium *Pseudomonas* sp. strain ACP (Figure 2.5). None of the other ACC degrading microorganisms gave any PCR products. This implies that none of these microbes had genes with high enough homology to the previously sequenced ACC deaminase genes for the designed primers to bind and allow PCR amplification.

Southern blot analysis of genomic DNA hybridised to an ACC deaminase gene probe was performed to rule out the possibility that although PCR primers did not bind to ACC deaminase gene sequences, there was homology to sequence in other regions of the ACC deaminase gene. The probe was an 817-bp fragment from the ACC deaminase gene of *Pseudomonas* sp. strain ACP. Results of this analysis indicated that only *Pseudomonas* sp. strain 17 has an ACC deaminase gene that is homologous to that of *Pseudomonas* sp. strains 65G and ACP (Figure 2.6). Furthermore, *Pseudomonas* sp. strain 17 appears to have 2 copies of the ACC deaminase gene as two bands were present on the Southern blot. As both of these bands are much bigger than the probe, and sequencing studies indicated that this gene does not have a *PvuII* site (Chapter III), it is not possible that these bands represent fragments of the same ACC

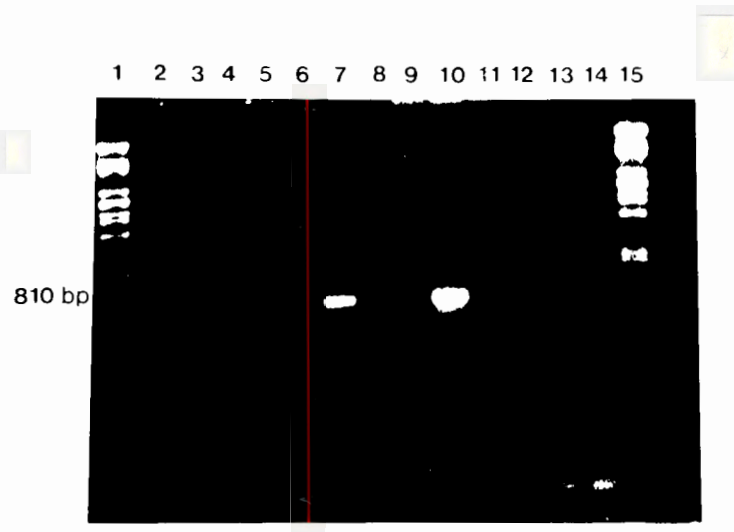


Fig. 2.5 Agarose gel electrophoresis of ACC deaminase gene PCR products.

Lanes 1 and 15, λ DNA digested with *Pst*I; lanes 2-10 represent PCR reactions with *Pseudomonas* 1, 2, 5, 12, 15, 17, 18, TL6 (negative control) and ACP (positive control) template DNA respectively; lanes 11-13, PCR reactions with yeast 6, 14 and *Hansenula saturnus* genomic DNA; lane 14, water control.

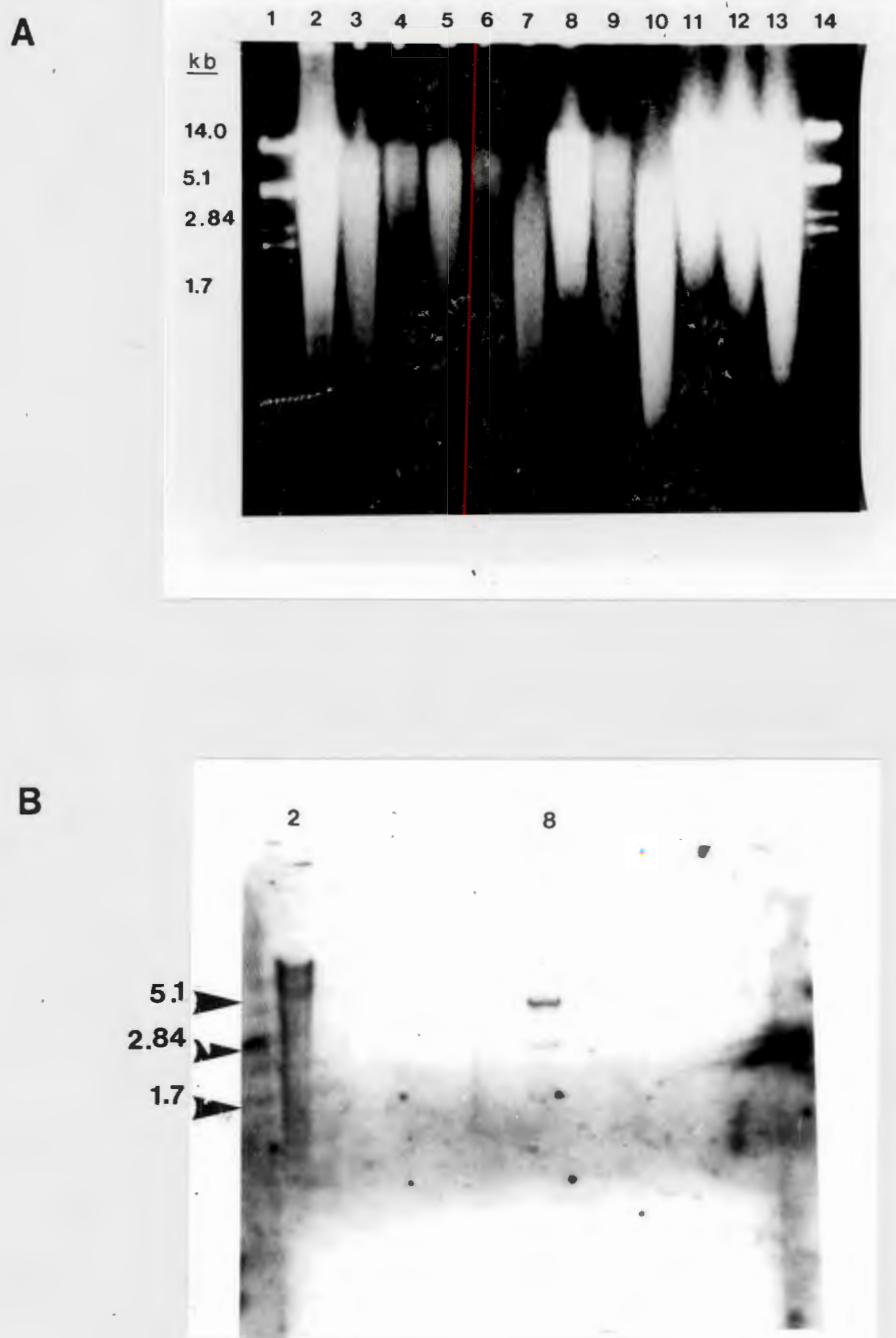


Fig. 2.6 (A) Agarose gel and (B) Southern blot of genomic DNA from ACC degrading microorganisms.

The probe was an 817-bp DIG-labelled fragment of the ACC deaminase gene from *Pseudomonas* sp. strain ACP. Lanes 2 to 13 each contain 5 μ g genomic DNA digested with *PvuII*. Arrows indicate bands on the Southern blot. Lane 1, molecular size marker, λ DNA digested with *PstI*; lanes 2 to 13 contain the positive control *Pseudomonas* sp. ACP, *Pseudomonas* 1, 2, 5, 12, 15, 17, 18, the negative control TL6, Yeasts 6 and 14, and *H. saturnus* respectively.

deaminase gene.

This confirms the results obtained from PCR amplification viz. that only *Pseudomonas* sp. strain 17 has an ACC deaminase gene homologous to that of *Pseudomonas* sp. strain ACP and *Pseudomonas* sp. strain 65G. All other ACC degrading microorganisms have novel ACC deaminase genes. However, caution needs to be heeded as all hybridisation steps were performed at 65°C. Lowering the temperature of the hybridisation incubations would decrease the stringency of the reaction and allow for binding of less homologous genes to the ACC deaminase gene probe. Lower hybridisation incubations may yield results which show that ACC degrading microorganisms have a gene distantly related to the ACC deaminase gene of *Pseudomonas* sp. strain ACP.

It was decided to clone the ACC deaminase genes from *Pseudomonas* sp. strains 15 and 17 (both bacteria belong to the species *Pseudomonas fluorescens*) as these organisms showed the highest ACC deaminase activity and strain 15 had a potentially novel ACC deaminase gene.

CHAPTER III

Isolation and Characterisation of Two *Pseudomonas fluorescens* ACC Deaminase Genes

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3.1 SUMMARY

Genomic DNA libraries of two *P. fluorescens* strains displaying the highest ACC deaminase activity (strains 15 and 17) were constructed in the suicide vector pEcoR251. Screening the libraries on minimal medium containing ACC as the sole nitrogen source resulted in the isolation of colonies carrying plasmids conferring ACC degrading ability. One plasmid from each library, pBC15 (strain 15) and pBC17 (strain 17), was chosen for further studies.

DNA sequence analysis of a pBC17 *PvuII* subclone revealed a single open reading frame (ORF) of 1017 nucleotides. This ORF was highly homologous to previously sequenced ACC deaminase genes. Subcloning pBC15 revealed that the ACC deaminase gene was contained on a 1.5 kb *BamHI*-*BglIII* fragment. Preliminary end sequencing studies indicated that this gene is very different from previously sequenced ACC deaminase genes.

3.2 INTRODUCTION

Prior to this study, two independent research groups isolated and characterised ACC deaminase genes. Sheehy *et al.* (1991) determined the amino acid sequence of the ACC deaminase from *Pseudomonas* sp. strain ACP. They then used this sequence to design degenerate oligonucleotide primers to PCR-amplify a 370-bp fragment of the ACC deaminase gene from this organism. This fragment was used as a probe to screen a *Pseudomonas* sp. strain ACP genomic DNA library in λ ZAP. Southern analysis revealed six positive plaques. Two of these were rescreened with antibodies raised against ACC deaminase and one plaque reacted with the antibody.

Klee *et al.* (1991) used a simpler method of isolating an ACC deaminase gene. They constructed a cosmid library from genomic DNA of the ACC degrading microorganism *Pseudomonas* sp. strain 65G. They screened *E. coli* cells containing cosmids for their ability to grow on minimal medium with ACC as a sole nitrogen source.

Our aim was to clone the ACC deaminase genes from *P. fluorescens* strains 15 and 17 using a combination of these methods.

3.3 MATERIALS AND METHODS

Standard methods and materials are recorded in Appendix A and B. Plasmid maps of cloning vectors are shown in Appendix C.

3.3.1 Bacterial strains, plasmids and culture conditions

P. fluorescens strains 15 and 17 were grown and maintained in LB broth or YPD medium. Cultures were stored in 0.1 M MgSO₄ in Eppendorf tubes at 4°C.

E. coli strains used for all DNA manipulations are presented in Table 3.1. Genomic DNA fragments were cloned into pEcoR251 (Zappe *et al.* 1986), and subcloned into the Bluescript vector pSK (M13-) (Stratagene). The plasmid pSK was used for exonuclease shortening and nucleotide sequencing of *P. fluorescens* DNA fragments.

Table 3.1 *E. coli* strains used in this study

<u><i>E. coli</i> strain</u>	<u>Genotype</u>	<u>Reference</u>
JM105	<i>thi, ppsL, endA, sbcB15, hspR4, Δ(lac-proAB) [F', traD36, proAB, lacI^qZΔM15]</i>	Yanish-Perron <i>et al.</i> (1985)
JM109	<i>recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, λ⁻, Δ(lac-proAB), [F', traD36, proAB, lacI^qZΔM15]</i>	Yanish-Perron <i>et al.</i> (1985).
DH5α	<i>F⁻, recA1, endA1, gyrA96, thi-1, hsdR17 (r_K⁻, m_K⁺), supE44, relA1, deoR Δ(lacZYA-argF)U169, ϕ80dlacZΔM15</i>	Hanahan (1983)

3.3.2 Construction and screening of genomic DNA libraries

Genomic DNA from the ACC degrading *P. fluorescens* strains 15 and 17 was prepared according to the method of Klee *et al.* (1991). DNA (10mg) from each *Pseudomonad* was partially digested with the restriction endonuclease *Sau3A* and size fractionated on a 10% to 40% sucrose gradient. Fractions containing fragments between 5 kb and 11 kb were pooled, ethanol precipitated and resuspended in TE, pH 8.0. DNA fragments were cloned into the *BglIII* site of the positive selection vector pEcoR251 and transformed into competent *E. coli* JM105 cells according to the method of Draper *et al.* (1989).

E. coli JM105 transformants were screened for ACC deaminase activity on M9 minimal medium containing 3 mM ACC as the sole nitrogen source. The plates were incubated for 3 days at 37°C. Positive clones were further screened for the presence of the ACC deaminase gene by subjecting cell free extracts to ACC deaminase assays (Section 2.3.4).

3.3.3 Plasmid DNA manipulations and sequencing

Plasmid DNA was routinely prepared by the alkali hydrolysis method of Ish-Horowicz and Burke (1981). Both small scale (mini prep) and large scale isolation of plasmid DNA are detailed in Appendix A.

Recombinant pEcoR251 derivatives were digested with restriction endonucleases, and insert fragments were ligated into compatible enzyme sites of the pSK vector polylinker. Plasmid DNA was transformed into *E. coli* JM109 or DH5 α cells. White colonies, indicative of insertional inactivation of the β -galactosidase gene of the vector (Viera and Messing 1982) were selected on LB plates containing X-gal. These colonies were screened for the presence of the ACC deaminase gene by growth on M9 minimal medium plates where ACC was the sole nitrogen source.

Plasmids with ACC degrading ability were shortened using exonuclease III according to the method of Henikoff (1984). These subclones were digested to completion with the endonuclease *SacI* that generates a 3' overhang which is not susceptible to

exonuclease III, and *Not I* that generates a 5' overhang which is susceptible to digestion by the enzyme. Exonuclease III digestion produced a range of unidirectionally shortened nested deletions for nucleotide sequencing by an adaption of the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977). Reactions were performed with the Sequenase^R Version 2.0 Sequencing kit and M13 forward and reverse primers. Two oligonucleotide primers complementary to internal regions of the ACC deaminase gene, used for PCR analysis (section 2.3.7), were also used in sequencing reactions. A detailed description of the Sequenase^R method with modifications is given in Appendix A. Compilation and analysis of genomic sequences was done on a GCG package version 8.0. This package was run on a DEC/VAX 6000-330 mainframe computer.

3.4 RESULTS AND DISCUSSION

3.4.1 Screening the genomic DNA libraries

E. coli JM105 derivatives harbouring plasmids with either *P. fluorescens* strain 15 or 17 DNA inserts were plated onto M9 minimal medium plates containing ACC as the sole nitrogen source and ampicillin (100 mg/ml). This procedure identified 10 positive colonies from the strain 15 library and 7 positive clones from the strain 17 DNA library. Six ACC degrading colonies, containing plasmids p15E, p15(5), and pBC15, and p17(i), p17(ii) and pBC17 respectively, which showed the most vigorous growth were subjected to ACC deaminase enzyme assays.

Clones pBC15 and pBC17 were chosen for all further studies. It is interesting that the specific activities of these clones were found to be much lower than those of the parental *Pseudomonads* (Figure 3.1); 6×10^{-3} and 1.3×10^{-1} U/mg; 11.74×10^{-3} U/mg and 1.2×10^{-1} U/mg respectively. A possible reason for these low specific activity values could include poor transcription initiation due to the insert being transcribed from its own promoter. This promoter may not be recognised efficiently by *E. coli* RNA polymerase and other transcription factors. Although Holtel *et al.* (1994) demonstrated that the *xyl* promoters, Pu and Ps, work as effectively in *E. coli* as they do in the native organism *Pseudomonas putida*, a number of *Pseudomonas* promoters are not recognised by *E. coli* RNA polymerases. The promoter of the endotoxin A gene from *P. aeruginosa* did not function in *E. coli* (Chen *et al.* 1987). Furthermore when the branched-chain keto acid dehydrogenase (*bkd*) gene promoter from *P. putida* was cloned in front of the streptomycin resistance gene, transformed *P. putida* cells could grow on medium containing streptomycin, but *E. coli* cells could not, indicating that *E. coli* does not read the *bkd* promoter well (Madhusudhan *et al.* 1990).

Although the colonies displayed low ACC deaminase activity, the bacteria grew well on ACC plates and were comparable to the growth of the parental *Pseudomonads* on DF media containing ACC as the only nitrogen source. This implies that very low concentrations of enzyme are necessary for growth on ACC and that *P. fluorescens* strains produce ACC deaminase in excess. This might be important in their natural

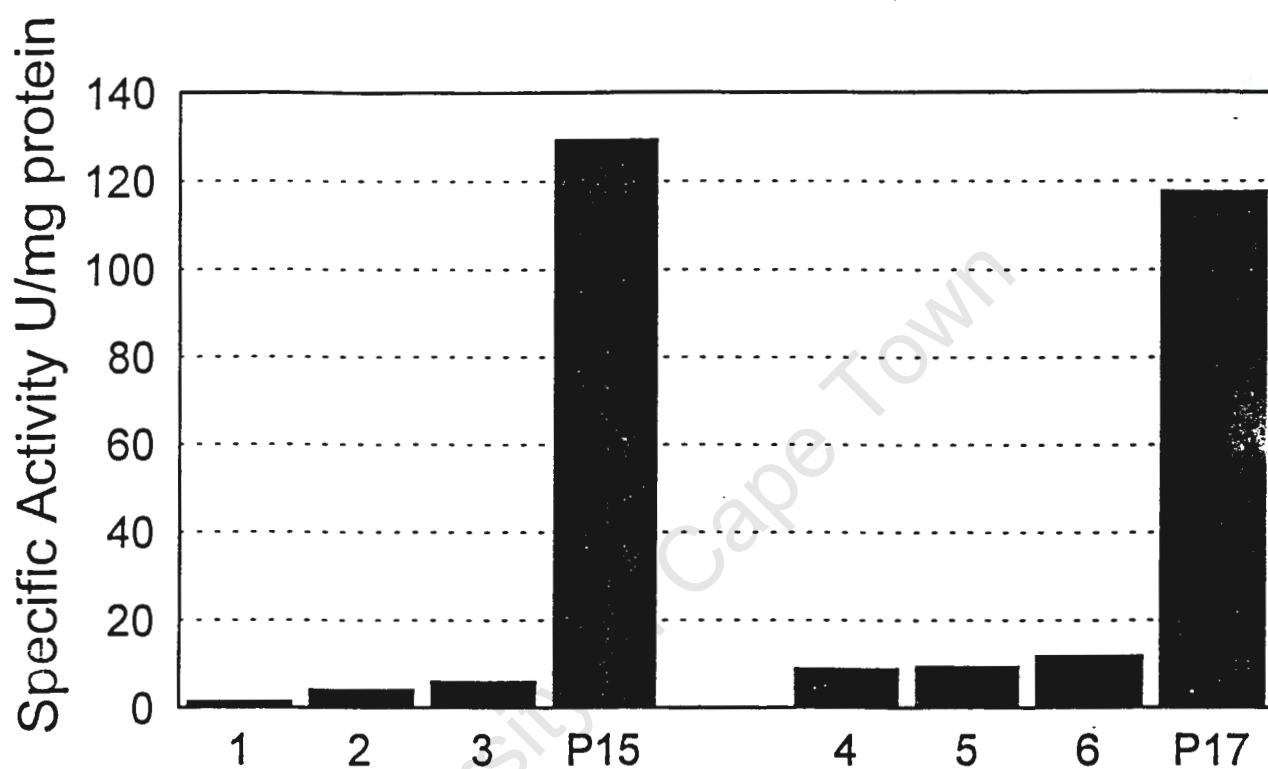


Fig. 3.1 ACC deaminase activity of ACC degrading colonies containing DNA from either *P. fluorescens* strain 15 or strain 17.

One unit of ACC deaminase activity is defined as the activity forming 1 μ mol of α -ketobutyrate (Honma and Shimomura 1978). Numbers 1-6 represent colonies harbouring plasmids p15(E), p15(5), pBC15, p17(i), p17(ii), and pBC17 respectively. P15=*P. fluorescens* strain 15; P17=*P. fluorescens* strain 17.

environment where ACC would not necessarily be concentrated in one small area as is the case with medium contained in a petri dish.

3.4.2 Subcloning pBC17

Based on the restriction map of pBC17 (Figure 3.2) subclonings shown in Figure 3.3 were carried out. pBC17 derivatives were tested for the presence of the ACC deaminase gene by PCR amplification with ACC deaminase specific primers (section 2.3.7), Southern blot analysis (section 2.3.8), ACC deaminase assays (section 2.3.4) and/or growth on M9 minimal-medium plates.

pSN2 was the only subclone to grow on ACC plates. PCR analysis confirmed that this clone contained the ACC deaminase gene (Figure 3.4). Preliminary end sequencing studies of pSN2 revealed that the forward primer did not bind to the plasmid DNA. Restriction endonuclease digests confirmed that a small region 5' to the *SmaI* site had been deleted. However the *PvuI* site approximately 100 bp upstream from the insert was still present (see Figure 3.5 for a plasmid map of pSN2).

To obtain the smallest insert containing the entire ACC deaminase gene, a Southern blot was carried out. This indicated that the gene was present on *PvuI*, *PvuII* and *ScaI/SalI* fragments of the pSN2 insert (Figure 3.6). As the *Pvu II* fragment was the smallest, it was cloned into the *EcoRV* site of pSK in both orientations to produce plasmids pBP1 and pPB3. These plasmids were shortened with exonuclease III to generate a range of nested deletions which were used for nucleotide sequencing of the ACC deaminase gene.

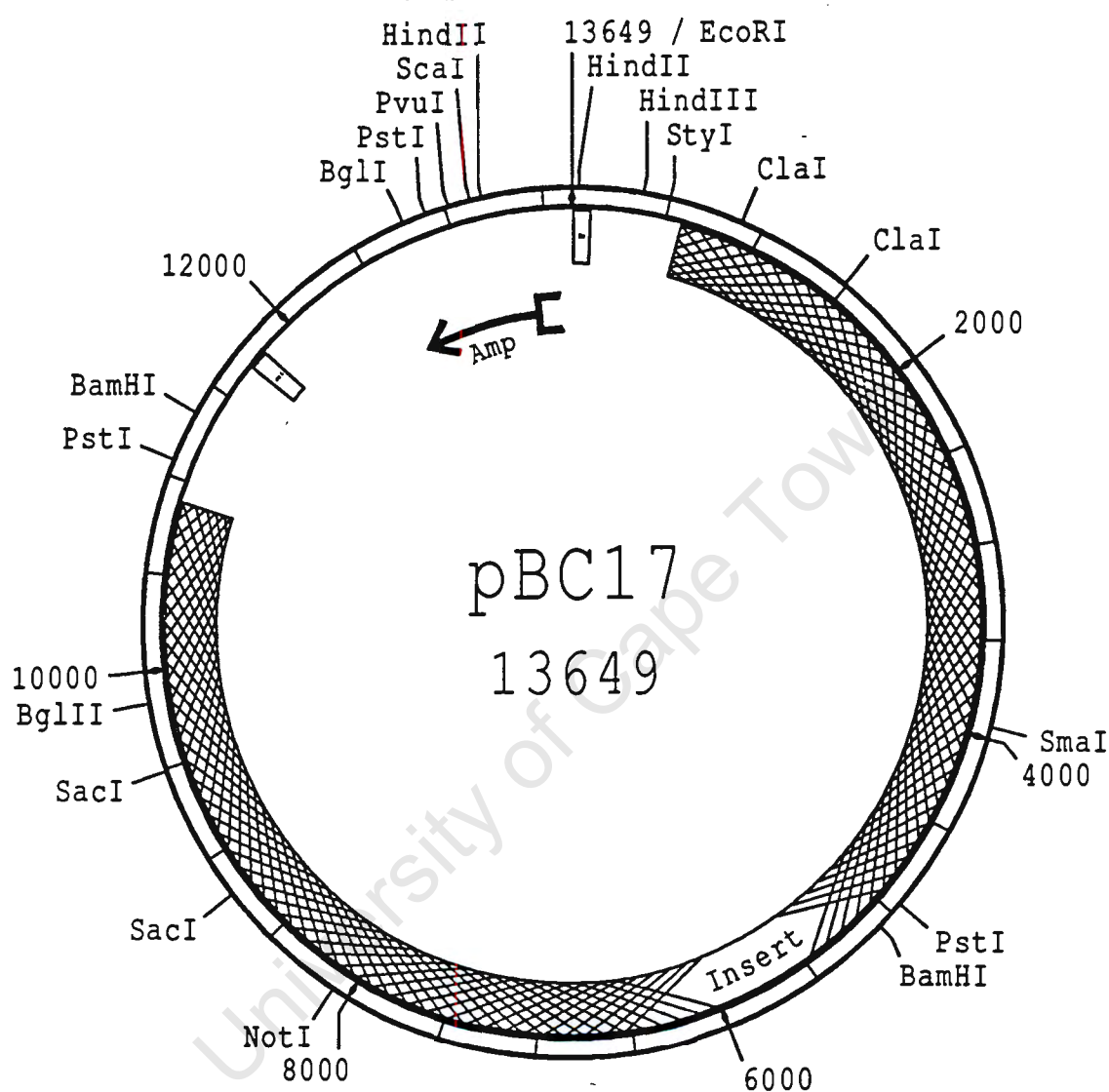
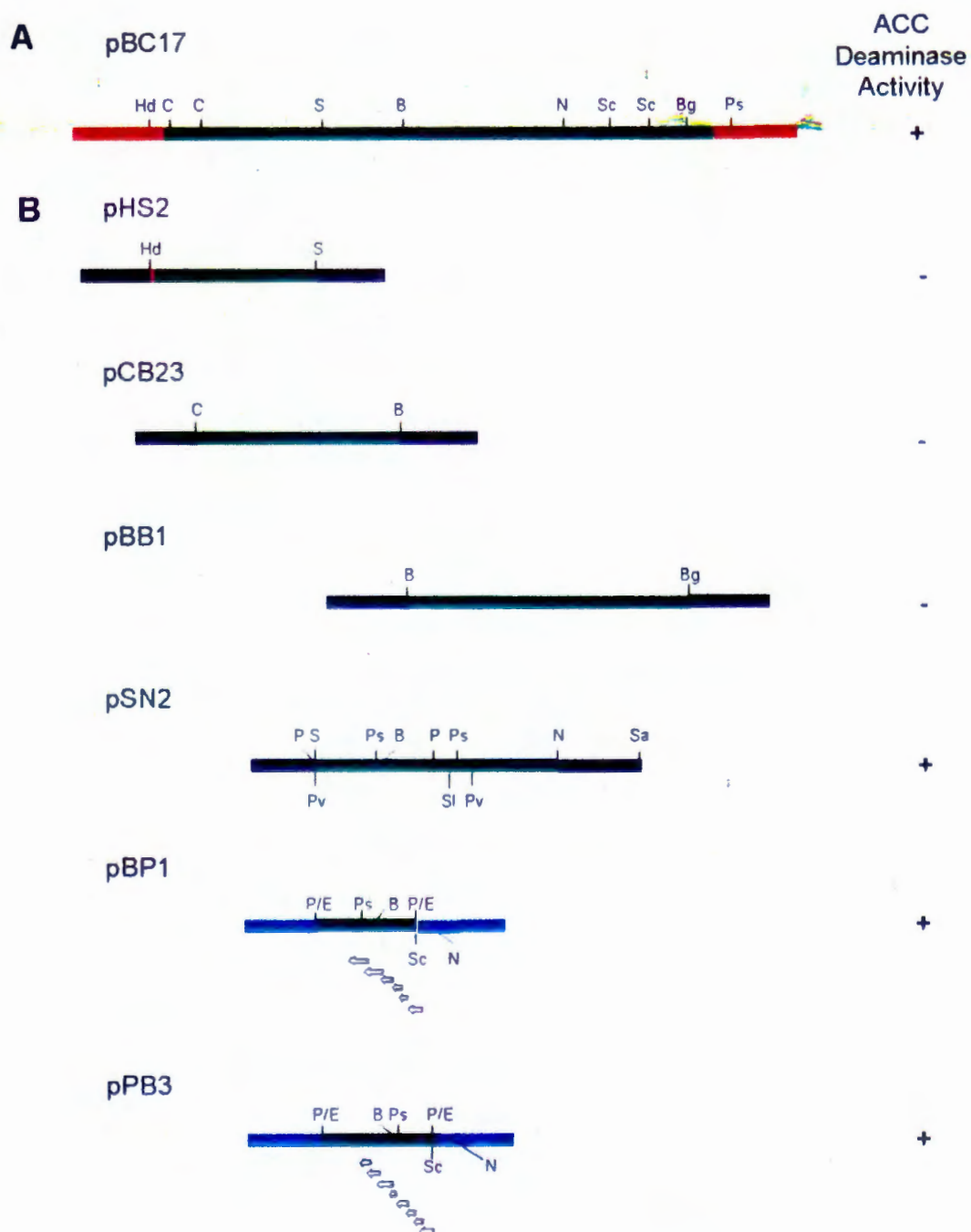


Fig. 3.2 Restriction endonuclease map of pBC17

The relative positions of the *P. fluorescens* strain 17 DNA insert, the β -lactamase gene (Amp^r), the λ_{PR} promoter, the origin of replication (ori) and the recognition sites of various restriction endonucleases are shown.



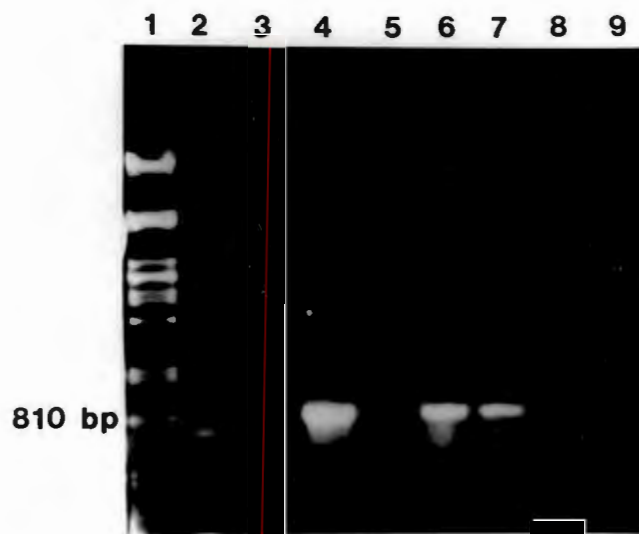


Fig. 3.4 PCR analysis of pBC17 subclones

ACC deaminase gene specific primers were used to amplify fragments from pBC17 subclones. Lane 1 molecular size markers, λ DNA digested with *Pst* I; lanes 2, 3, 4 and 5, PCR reaction products of pBB1, pCB23, pSN2 and pHS2 respectively; lanes 6 and 7, PCR products of the positive controls pBC17 and *P. fluorescens* strain 17; lanes 8 and 9, negative controls of PCR reactions containing no DNA, and no reagents respectively.

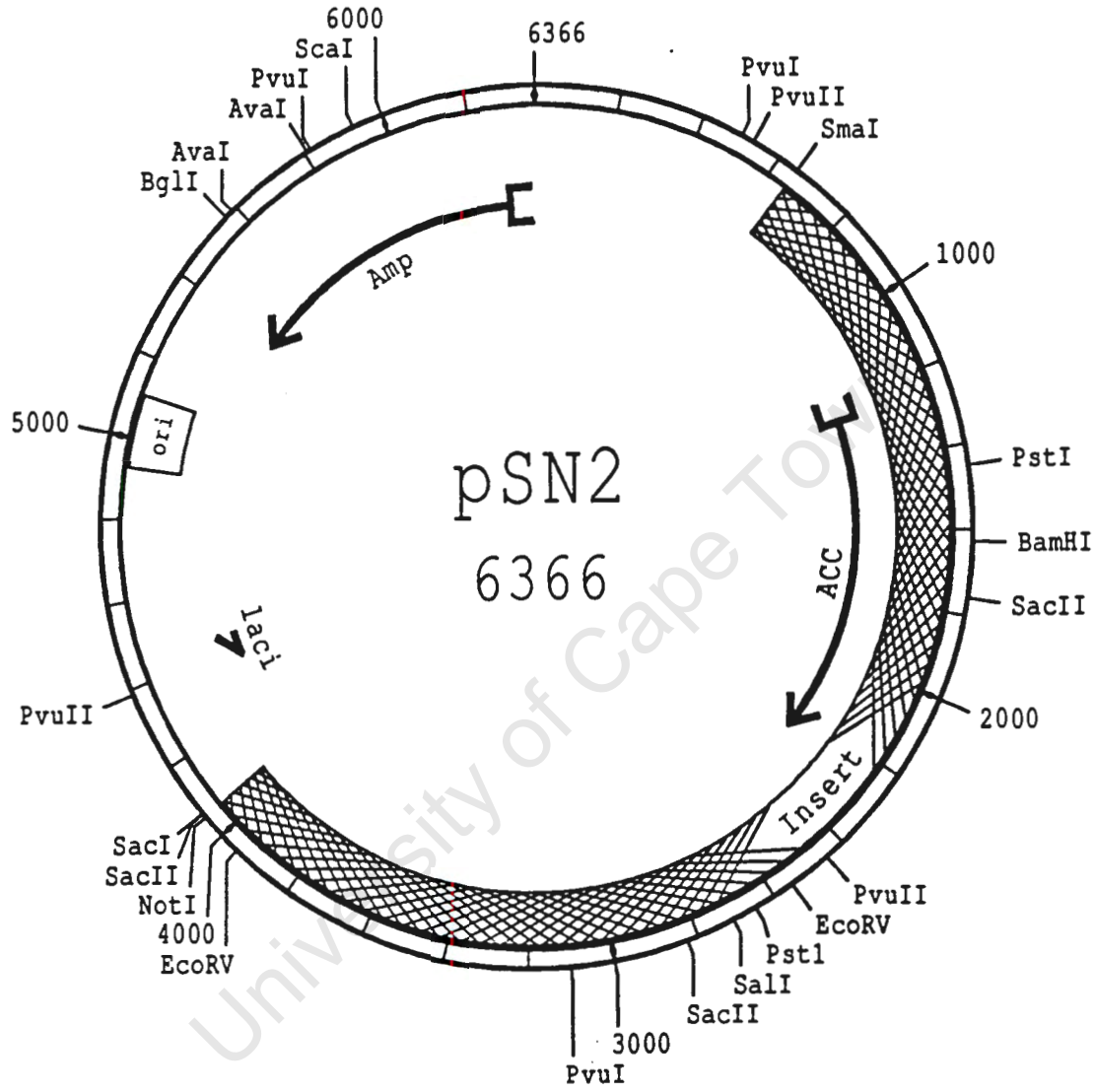


Fig. 3.5 Restriction endonuclease map of pSN2.
Relative positions of the pBC17 derived insert, the β -lactamase gene (Amp), the *lac i* promoter, the origin of replication (ori) and the ACC deaminase gene (ACC) are shown.

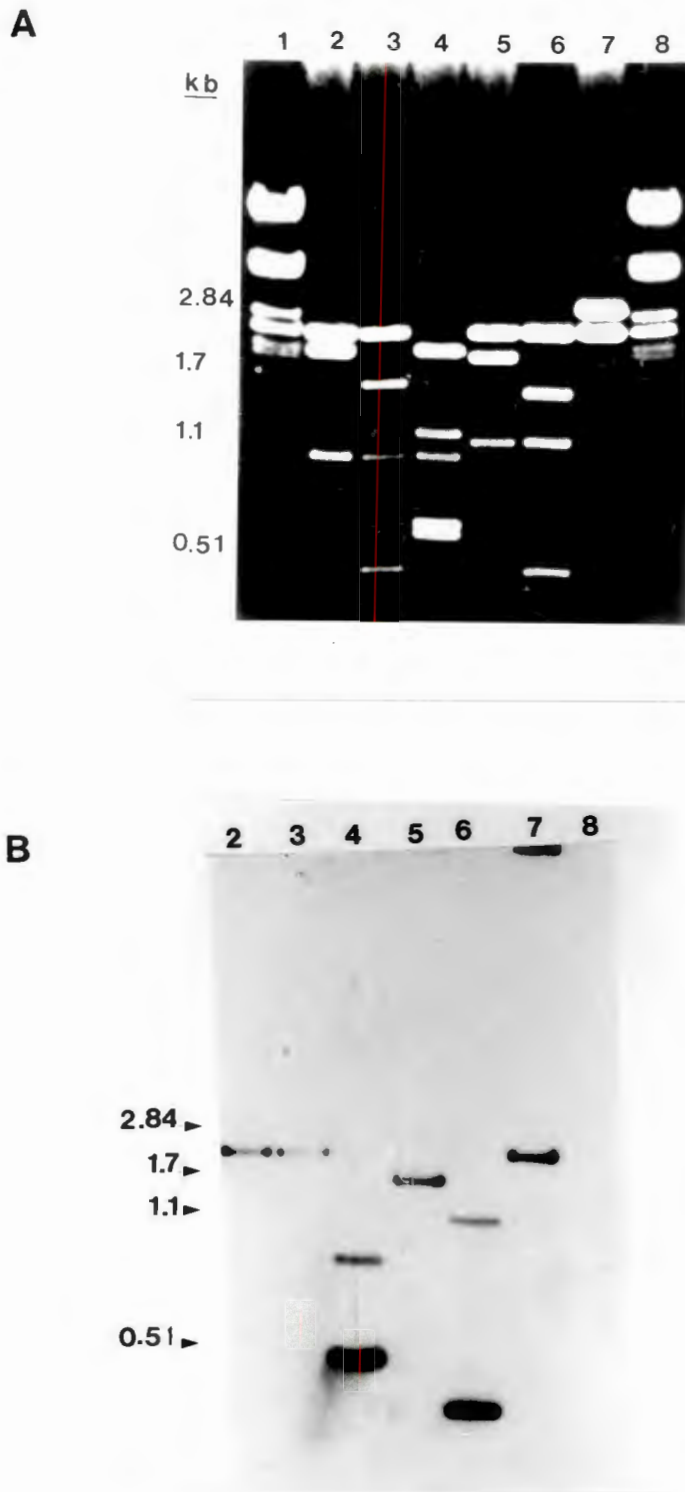


Fig. 3.6 (A) Agarose gel and (B) Southern blot of pSN2 digested with various restriction endonucleases.

The probe was an 817-bp DIG-labelled fragment of the *Pseudomonas* sp. strain ACP ACC deaminase gene. Lanes 2 to 7 each contain 500 ng of pSN2 digested with *PvuI*, *PvuI* and *NotI*, *PvuI* and *PstI*, *PvuII*, *PvuII* and *BamHI*, and *ScaI* and *SalI* respectively. Lanes 1 and 8 contain molecular size markers, λDNA digested with *PstI*.

3.4.3 Nucleotide sequence analysis of the *P. fluorescens* strain 17 ACC deaminase gene

Initially sequencing the strain 17 ACC deaminase gene proved to be problematic because of the high GC ratio of the sequence. For this reason, the Sequenase[®] 2.0 DNA sequencing kit was used, in which the DNA polymerase is stabilised by glycerol. This allows one to increase priming reaction temperatures from 40°C to 65°C, and to terminate sequencing reactions at 48°C as opposed to 37°C. The higher temperatures decrease the chance of secondary structure formation of the denatured DNA template. However, this did not produce a readable DNA sequence. Therefore, single stranded binding protein (SSB) was added to the sequencing reaction. These bind cooperatively to single stranded DNA, but not to double stranded DNA. SSBs are effective in sequencing as they accelerate the reannealing of complementary polynucleotides and increase the processivity of DNA polymerases by removing intrastrand secondary structures that form barriers to the progression of these enzymes (Sambrook *et al.* 1989). The addition of SSB to *P. fluorescens* strain 17 templates improved DNA sequencing reactions remarkably by minimising secondary structure and compressions, and increasing the chain length of the product.

A number of overlapping fragments of the 1.9 kb insert of pBP1 and pPB3 were sequenced. A contiguous sequence of 1164 bp was obtained by assembling the sequence of these fragments (Figure 3.7). The ACC deaminase gene ORF is 1017 bp long and stretches from nucleotides 83 to 1099. A possible Shine-Dalgarno consensus ribosome binding sequence (Von Heijne 1984) is located at position -13 in relation to the ATG corresponding to the first methionine found in the protein sequence. The gene sequence ends with a TGA termination codon. 3' sequence analysis with the GCG programme "terminator" did not identify any prokaryotic factor independent RNA terminators. Likewise, the GCG programme "repeat" did not reveal any significant direct repeat sequences in the regions 5' and 3' to the gene.

Homology comparisons with other ACC deaminase genes revealed that the strain 17 gene is 86.8% similar to that of *Pseudomonas* sp. strain 65G, and

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1  GCCCCCTCTGAAAGCACCTGGAACGCAGCCTTTTTTGCCTTTGGGTCTGGCGCTTGGATGT  60
61  CCAATAAACAAGGAGCAGAGTCATGAACCTGAATCGTTTTAAACGTTATCCGCTGACCTT  120
      M N L N R F K R Y P L T F
121  CGGTCCTTCTCCGATCACGCCCTTGAAACGCCTCAGCGAACACCTGGGCGGTAAGGTCGA  180
      G P S P I T P L K R L S E H L G G K V E
181  GTTGTATGCCAAACGTGAAGACTGCAACAGTGGCCTTGGCGGCAACAAGACGCG  240
      L Y A K R E D C N S G L A F G G N K T R
241  CAAGCTCGAATACCTGATTCCCGAGGCGCTCGAACAGGGCTGCGACACCTTGGTGTCCAT  300
      K L E Y L I P E A L E Q G C D T L V S I
301  CGGCGGGATCCAGTCGAACCAGACCCGCCAGGTGCGCGCTGTCGCTGCCACCTCGGTAT  360
      G G I Q S N Q T R Q V A A V A A H L G M
361  GAAGTGTGTGCTGGTCCAGGAAACTGGGTGAACTACTCCGACGCCGTGTATGACCGGGT  420
      K C V L V Q E N W V N Y S D A V Y D R V
421  CGGCAATATCGAGATGTCCCGCATCATGGGGCTGACGTACGACTGGACGCTGCAGGGTT  480
      G N I E M S R I M G A D V R L D A A G F
481  CGACATCGGTATTCCGGCCAGTTGGGAGAAGGCCATGAACGACGTGGTGAACGGGGTGG  540
      D I G I R P S W E K A M N D V V E R G G
541  CAAGCCGTTCCCGATACCGCGGGTTGTTCCGAACATCCCTACGGCGGGCTTGGGTTCGT  600
      K P F P I P A G C S E H P Y G G L G F V
601  CGGCTTTGCCGAGGAAGTGCGGGAACAGGAAAAACAACCTGGGGTTCAAGTTCGACTACAT  660
      G F A E E V R E Q E K Q L G F K F D Y I
661  CGTGGTCTGCTCCGTGACCGGCAGTACCCAGGCCGGCATGGTCTGCTCGGTTTTGCCGCGGA  720
      V V C S V T G S T Q A G M V V G F A A D
721  CGGCCGTTTCAAGAACGTTATCGGCATTGATGCCTCGGCCAAGCCCGAGAAGACCAAGGC  780
      G R S K N V I G I D A S A K P E K T K A
781  ACAGATCCTGCGTATCGCCCGGCACACCGCCGAGCTGGTGGAACTGGGGCGTGAGATCAC  840
      Q I L R I A R H T A E L V E L G R E I T
841  CGAAGACGACGTGGTGTCTGATACACCGTTTCGCCTACCCGGAATATGGTTTGGCCCAACGA  900
      E D D V V L D T P F A Y P E Y G L P N E
901  AGGTACGCTGGAAGCCATTTCGCCTGTGCGGCAGCCTTGAAGGTGTGTTGACCGACCCAGT  960
      G T L E A I R L C G S L E G V L T D P V
961  GTATGAAGGCAAATCCATGCACGGGATGATCGAAATGGTCCGCCGTGGCGAGTTCCCCGA  1020
      Y E G K S M H G M I E M V R R G E F P E
1021  AGGCTCGAAAGTGCTTTACGCGCACCTGGGTGGGGCACCTGCGCTGAACGCCTACAGCTT  1080
      G S K V L Y A H L G G A P A L N A Y S F
1081  CCTGTTCCGCAACGGCTGATTAAACGCCAATAACCTGTGGGAGGGCGCTTGCTCTGAAGCG  1140
      L F R N G *
1141  GTGGGTGAGTCGGCATCAATATTG  1164

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Fig. 3.7 Nucleotide sequence of *P. fluorescens* strain 17 ACC deaminase gene and adjacent regions.

The derived amino acid sequence is presented. The Shine-Dalgarno ribosome binding sequence is underlined. The termination codon is represented by an asterisk.

71.7% similar to that of strain ACP. Sequence alignments of these three ACC deaminase genes are shown in Figure 3.8. It is interesting to note that there is no homology between the three ACC deaminase genes downstream from the stop codon. Strain ACP contains multiple direct repeats and an inverted repeat downstream from the gene which are potentially capable of forming a stem loop structure, and might act as a transcription termination signal (Sheehy *et al.* 1991). However, if this region were essential to termination one would expect to find it downstream from all three ACC deaminase genes. There is also little homology between the sequences upstream from the ATG start codon. The only area of significant homology is the AAGGA Shine-Dalgarno ribosome binding site.

Amino acid alignments indicate that *P. fluorescens* strain 17 ACC deaminase is 97% similar to the *Pseudomonas* sp. strain 65G protein and 82.2% homologous to the strain ACP protein (Figure 3.9). Most of the amino acid changes between the three proteins are conservative. This high degree of similarity between nucleotides of the ACC deaminase genes and their corresponding proteins suggests that this enzyme is highly specific for its substrate, and that certain amino acid changes may result in loss of activity.

3.4.4 Subcloning pBC15

A restriction map of pBC15 was generated (Figure 3.10). Subcloning of pBC15, as outlined in Figure 3.11, gave only one plasmid, pBG1, showing ACC deaminase activity. As subclone pSE5 lacks the 3' 1.445-kb *EcoRV/Bgl II* fragment present in pBG1, it was hoped that the ACC deaminase gene was partially deleted by the removal of this fragment. However, analysis of the 3' end sequence of pSE5 showed no homology to previously sequenced ACC deaminase genes, and very little homology to other sequenced *Pseudomonas* genes. This may suggest that the ACC deaminase gene is entirely contained on the deleted 1.445kb *EcoRV/Bgl II* fragment. However, 3' end sequencing of pBG1 indicated that this region also had no homology to known ACC deaminase genes.

	1				50
Pse65g	.a-a--c-at	-----	-----	-t-----	---g----
Psef17	.tg-c--ata	-a-----	-----	-----	---a----
Pseacp	tc---t--cg	-----	.t-c-----	-----c-a--	a--ccct---
Consensus	--TTTCAC--	ATCAAGGAGC	AGAGTCATGA	ACCTGAATCG	TTTT-AACGT
	51				100
Pse65g	-----at---	-----	-----c---	-----	-----
Psef17	-----	-----	-----g---	-----	---a-----
Pseacp	--c-----	-t-----g--	ga-g--a---	caa--gc-ag	c---t--g--
Consensus	TATCCGCTGA	CCTTCGGTCC	TTCTCC-ATC	ACGCCCTTGA	AGCGCCTCAG
	101				150
Pse65g	tc---t---	--g-----	-----	-----	-----
Psef17	-g-----	-----t---	-----t---	-----	-----
Pseacp	-a-g-----c	-----a---	-gc-t-----	---g-----c	-----
Consensus	C-AACACCTG	GGCGGCAAGG	TCGAGCTGTA	TGCCAAACGT	GAAGACTGCA
	151				200
Pse65g	-----	-----	--g-----	-----	-----c
Psef17	-----	-----c	-----	-----	-----
Pseacp	---c-----	---g-c---	-----	-a-----	-----t---
Consensus	ACAGTGGCCT	GGCCTTTGGT	GGCAACAAGA	CGCGCAAGCT	CGAATACCTG
	201				250
Pse65g	-----	--a---g--	a-----t	-----t-	-----
Psef17	-----g-	-----a-	-----	--ct-----	-----
Pseacp	--c--t---	---t-ct-	---t-----	---c-----	-g-----
Consensus	ATTCCCGAAG	CGCTCGA-CA	GGGCTGCGAC	ACGCTGGTGT	CCATCGGCGG
	251				300
Pse65g	-----	-----	-t-----	t-----	-----t---
Psef17	g-----	-----	-----	c--t-----	-----c-
Pseacp	---t-----	-----g-	-----g-	g---g-g-	--t--t---
Consensus	CATCCAGTCG	AACCAGACCC	GCCAGGTCGC	-GCCGTCGCT	GCCCACCTGG
	301				350
Pse65g	-----	---t-----	-----	-----	-----g
Psef17	-t-----	t-----c	-----	-----	c-----c
Pseacp	-----	-----	---g-----	---c-----	---g-----a
Consensus	GCATGAAGTG	CGTGCTGGTG	CAGGAAAACT	GGGTGAACTA	TTCCGACGC-
	351				400
Pse65g	-----	---a-----	-----	---g-----	-----
Psef17	-----	-g-----	t-----	--c-----	---g-----
Pseacp	--c--c-----	-----	---c-----	-----tc	-c-----c-
Consensus	GTGTATGACC	GCGTCGGCAA	CATCGAGATG	TCGCGCATCA	TGGGCGCTGA
	401				450
Pse65g	---g--g--t	-----t-	-----t-	t--c-----	--a-----
Psef17	c--a--a--g	-----t-a-	-g-----	-----	---c-----
Pseacp	---t--c--c	-tgc--ac-	-----	---t-----c	agg--c----
Consensus	TGT-CG-CT-	GACGCCGC-G	GCTTCGACAT	CGGTATTTCGG	CC-AGTTGGG
	451				500
Pse65g	-a-----	--g---t--c	-----a--	-----a--	-----
Psef17	-----	-----	-----g-	-----	---c-----a
Pseacp	---g-t--gc-	-g-aag----	cg--cggcc-	-c-----	---a-g-----
Consensus	AGAAGGCCAT	GAACGACGTG	GTGGAAC-GG	GTGGCAAGCC	GTTTCCGATT
	501				550
Pse65g	---a-----	-----g--	-----t---	---c-----	-----
Psef17	-----	-t-----a-	-----	--g--t--g-	-----
Pseacp	-----a--c	---g--t---	c--gct----	-----g---	-----
Consensus	CCGGCGGGTT	GCTCCGA-CA	TCCCTACGGC	GGCCT-GGTT	TCGTCGGCTT

	551				600
Pse65g	-----a---	-----c---	-----g---	-----t---	
Psef17	-----a---	-----a---	-----ac---	-----g---	
Pseacp	c-g-----	-----c---	-----gcc---	-t-----	-a-----
Consensus	TGCCGAGGAG	GTGCGGGAGC	AGGAAAA-GA	ACTGGGCTTC	AAGTTCGACT
	601				650
Pse65g	-----g---	-----g---	-----t---	-----t---	
Psef17	-----g---	-----g---	-----c---	-----c---	
Pseacp	-tg---c---	g-t-----	-----c---	-----g---	-----g---
Consensus	ACATCGTGGT	CTGCTCCGTG	ACCGGCAGTA	CGCAGGCCCG	CATGGT-GT-
	651				700
Pse65g	-----g---	-----t---	-----t---	-----t---	
Psef17	-----t---	-g-----	-----t---	-----t---	-----c---
Pseacp	--c-----	-----c---	cg-cg-tcg-	-----g---	-----c---
Consensus	GGTTTCGCCG	CTGACGGCCG	TTCGAAGAAC	GTGATCGGCA	TCGATGCTTC
	701				750
Pse65g	-----g---	--a-----	-----t---	-----t---	
Psef17	-----g---	--a-----	-----t---	-----t---	-----g---
Pseacp	-----a---	-c-----gc	gc-ag-----	-acc-----	--ga-----g-
Consensus	GGCCAAGCCC	GAGCAGACCA	AGGCACAGAT	CCTGCGCATC	GCCCGACACA
	751				800
Pse65g	----t---t-	-----gt---	-----t---	g-----t---	
Psef17	----c---c-	-----a---	-----t---	-----c---	
Pseacp	----g---aa	a--c-gc---	-a-----t-	---t-cgg-c-	-----t---
Consensus	CCGC-GAG-T	GGTGGA-CTG	GGGCGCGAGA	TCACGGAAGA	CGACGTGGTG
	801				850
Pse65g	-----t---	-t-t-----	-----t---	-----c---	
Psef17	-----a---	cg-----	-----t---	-----t---	
Pseacp	-----cga-	-c-----ggg	t-----c-	-a-----g-	
Consensus	CTCGATACGC	G-TTCGCCTA	CCCGBAATAT	GG-TTGCCCA	ACGAAGGCAC
	851				900
Pse65g	at-----	-----a---	-----t---	-----t---	-----a---
Psef17	-----t---	-----t---	-----t---	-----t---	-----t---
Pseacp	-----g---	-----t---	---cgc--ac	g--g--a---	-----t---
Consensus	GCTGGAAGCC	ATCCGCCCTGT	GCGGCAGCCT	TGAAGGCGTG	CTGACCGACC
	901				950
Pse65g	-g-a-----	---t-----	-----t---	-----t---	-----t---
Psef17	-a-g-----	-----c---	-----g---	-----t---	-----t---
Pseacp	-c-c-c---	-----c---	-----t---	-----g---	---aac---
Consensus	C-GT-TATGA	AGGCAAATCG	ATGCACGGCA	TGATCGAAAT	GGTCCGCCGT
	951				1000
Pse65g	--t-----	-----t---	c-----t---	-----a---	-----t---
Psef17	-----g---	-----t---	-----t---	-----t---	-----t---
Pseacp	-----t---	-g-----	-cgc-----	-t-----	-c-c---t---
Consensus	GGCGAATTCC	CCGAAGGCTC	GAAAGTGCTT	TACGCGCACC	TGGGTGGGGC
	1001				1050
Pse65g	-----t---	-----t---	-----t---	t--t-----	-a-g--ta-a
Psef17	a--t-----	-----t---	-----t---	---c-----	---ttaac--
Pseacp	-----t---	-----g---	---ta-c---	---ag-----	---a---ctc-
Consensus	GCCGGCGCTG	AACGCCTACA	GCTTCCTGTT	CCG-AACGGC	TGA-CG--GC
	1051		1079		
Pse65g	actgcttt--	g--tcatct-	tg-----.		
Psef17	c-ataa-c--	tg-g-gg-c-	ctt-ct--.		
Pseacp	g-ccgg-ggc	c--c--cc-gc	aa-c---gt		
Consensus	-A----C-TG	-AG-A--G-G	--GGAGCT-		

Fig. 3.8 Alignment of the three sequenced ACC deaminase genes.
 A derived consensus sequence is shown below the alignment. Nucleotides identical to the consensus are indicated by dashes (-) and nucleotides differing from the consensus are shown in small lettering. The ACC deaminase genes represented are from *Pseudomonas* sp. strain 65G (Pse65g), *P. fluorescens* strain 17 (Psef17) and *Pseudomonas* sp. strain ACP (Pseacp).

	1				50
Pse65g		e		q	
Psef17		k		e	
Pseacp		q p	t q	a k	h
Consensus	MNLNRF-RYP	LTFGPSPITP	LKRLS-HLGG	KVELYAKRED	CNSGLAFGGN
	51				100
Pse65g		i			
Psef17					
Pseacp		a			
Consensus	KTRKLEYLIP	EALEQGCDTL	VSIGGIQSNQ	TRQVA AVAAH	LGMKCVLVQE
	101				150
Pse65g					s
Psef17					n
Pseacp		q	vp	d f r	d e s r a
Consensus	NWVNYSDAVY	DRVGNIEMSR	IMGADVRLDA	AGFDIGIRPS	WEKAM-DVVE
	151				200
Pse65g	q			q	
Psef17	r			e q	
Pseacp	a a	l		a a	
Consensus	-GGKPFPIPA	GCSEHPYGGL	GFVGFAEEVR	-QEKELGFKF	DYIVVCSVTG
	201				250
Pse65g					
Psef17			k		
Pseacp		adr	a	re t	q k g e
Consensus	STQAGMVVGF	AADGRSKNVI	GIDASAKPEQ	TKAQILRIAR	HTAELVELGR
	251				300
Pse65g					
Psef17		p			
Pseacp	mra	e g		art m	
Consensus	EITE-DVVLD	TRFAYPEYGL	PNEGTLAIR	LCGSLEGVLT	DPVYEGKSMH
	301				339
Pse65g					*
Psef17					*
Pseacp	n	r	v g	i d *	
Consensus	GMIEMVRRGE	FPEGSKVLYA	HLGGAPALNA	YSFLFRNG-	

Fig. 3.9 Amino acid alignment of three ACC deaminase proteins.

A derived consensus sequence is shown below the alignment. Amino acids differing from the consensus are shown in small lettering. ACC deaminases represented are from *Pseudomonas* sp. strain 65G (Pse65g), *P. fluorescens* strain 17 (Psef17) and *Pseudomonas* sp. strain ACP (Pseacp).

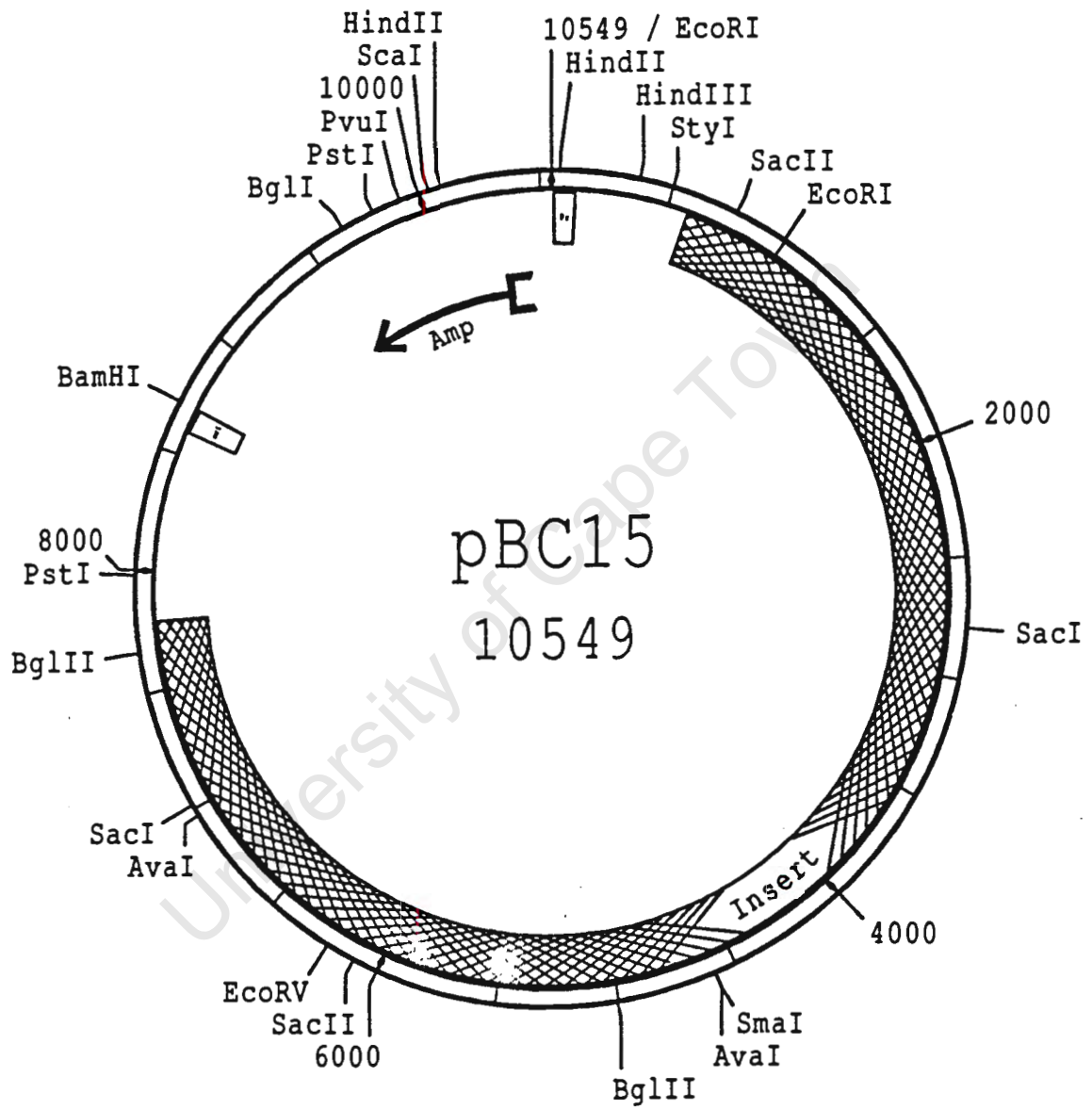


Fig. 3.10 Restriction endonuclease map of pBC15

The relative positions of the *P. fluorescens* strain 15 insert, the β -lactamase gene (*Amp*), the λ_{PR} promoter, the origin of replication (*ori*) and the recognition sites of various restriction endonucleases are shown.

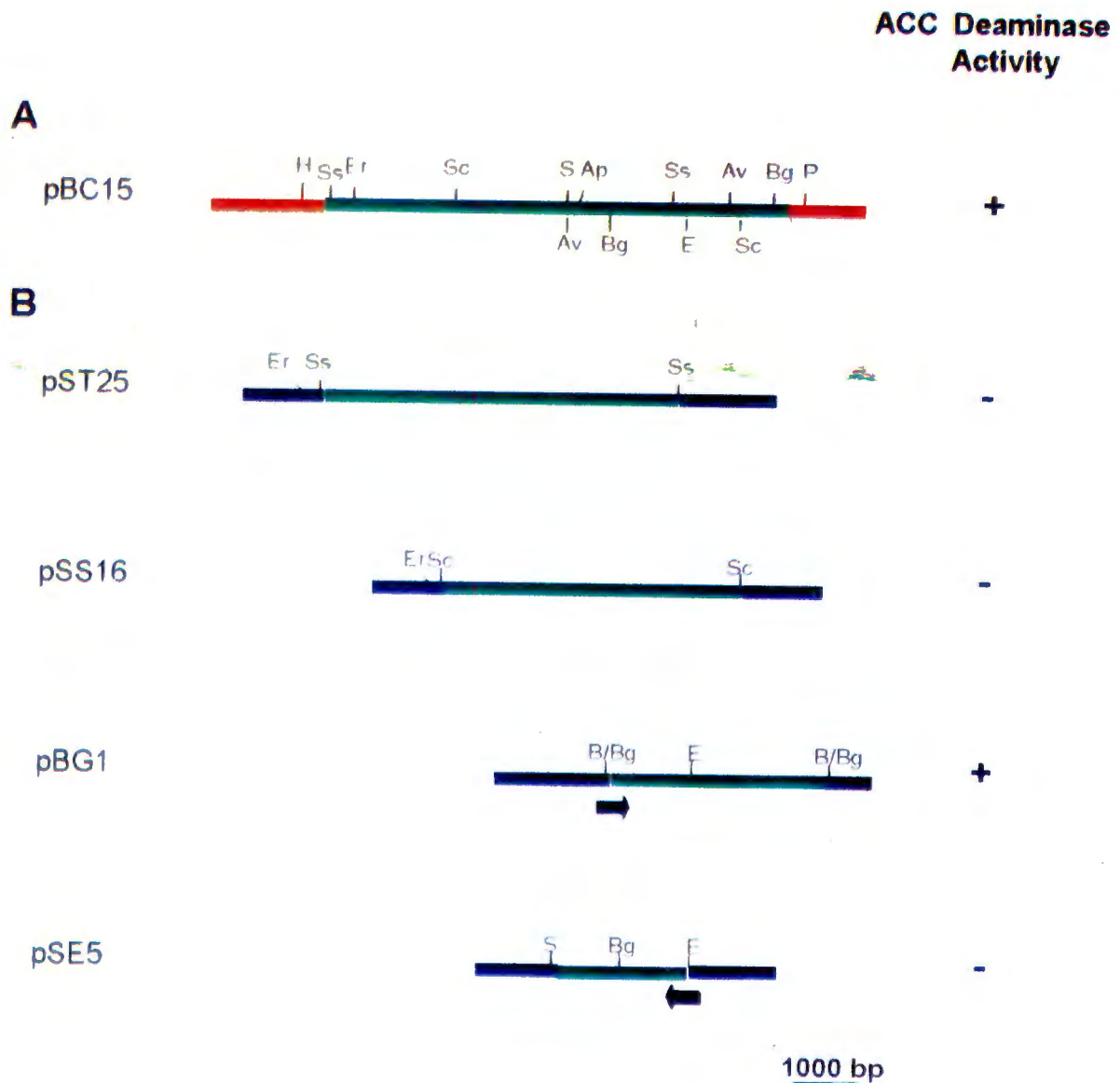


Fig. 3.11 Schematic diagram of DNA constructs containing *P. fluorescens* strain 15 DNA

Green lines correspond to *P. fluorescens* strain 15 DNA, red lines to pEcoR251 DNA, and blue lines to pSK DNA.

A. pBC15 contains 7.2 kb of insert cloned into the *Bgl* II site of pEcoR251.

B. pST25, pSS16, pBG1, and pSE5 contain the *Sst* II, *Sac* I, *Ava* I and *Bgl* II fragments of pBC15 respectively. All fragments were cloned into pSK.

Restriction sites of selected endonucleases are as follows: A, *Apal*; Av, *Ava* I; B, *Bam* HI; Bg, *Bgl* III; E, *Eco* RV; Er, *Eco* RI; H, *Hind* III; P, *Pst* I; S, *Sma* I; Sc, *Sac* I; Ss, *Sst* II. B/Bg is a *Bam* HI/*Bgl* III cloning junction. Arrows indicate regions of the clones that were end sequenced.

These data, together with the PCR (section 2.4.3) and Southern blot analysis (section 2.4.3) indicate that the ACC deaminase gene from *P. fluorescens* strain 15 is very different to previously sequenced ACC deaminase genes. Thus the sequencing of the 5' and 3' ends of the subclones will probably not reveal where the gene is on the insert. Therefore the entire insert of pBG1 must be sequenced to identify an ACC deaminase open reading frame.

CHAPTER IV

Cloning the *P. fluorescens* strain 17 ACC Deaminase Gene into a Plant Expression Vector

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4.1 SUMMARY

The *P. fluorescens* strain 17 ACC deaminase gene was PCR amplified and cloned into the primary expression vector pART7. The gene was placed between the CaMV 35S promoter and the *ocs-3'* termination sequence. This expression cartridge was then ligated into the T-DNA region of the binary vector pART27 in both orientations. *Agrobacterium tumefaciens* C58C1 was transformed with these recombinant plasmids. This will enable future development of transgenic plants and an assessment of the effect this ACC deaminase gene has on fruit ripening.

4.2 INTRODUCTION

4.2.1 *Agrobacterium* mediated gene transfer to plants

A. tumefaciens is a soil microorganism that causes crown gall disease in many dicotyledonous species. Crown gall occurs when a wound on the stem allows *A. tumefaciens* to invade the plant. After infection the bacteria cause cancerous proliferation of the stem tissue in the region of the crown.

The ability to cause crown gall disease is associated with the presence of the Ti (tumour inducing) plasmid within the bacterial cell. After infection, part of the Ti plasmid is integrated into the plant chromosomal DNA (Van Larebeke *et al.* 1974). This segment, the T-DNA, is flanked by 25 bp repeats which delimit the ends of the T-DNA and are important in DNA integration mechanisms (Zambryski 1992). Genes found in this region are responsible for oncogenic growth and the synthesis of opines, unusual amino acid derivatives, that *Agrobacterium* use as a nutrient supply. These genes are not essential to DNA transfer to plants.

The *vir* region of the Ti plasmid codes for proteins which sense and respond to the presence of phenolic compounds released by wounded plant cells. These proteins act in *trans* and are responsible for the transfer of T-DNA to the plant cell (Zambryski 1988). There are 22 *vir* genes on an octopine Ti plasmid which are present in 7 operons known as *vir A, B, C, D, E, G* and *H*.

4.2.2 Binary and cointegrate vectors

Because natural Ti plasmids cause disorganised growth, these vectors have been disarmed so that the T-DNA is non-oncogenic. Furthermore, Ti plasmids have been manipulated to create unique restriction endonuclease sites for the cloning of foreign genes. Two vector systems are in general use.

Cointegrate vectors are derivatives of a wild type Ti plasmid from which T-DNA encoded tumour genes have been replaced by pBR322 related cloning vector sequences. Foreign genes cloned into pBR322-derived intermediate vectors are

mobilised from *E. coli* into *A. tumefaciens*, and integrate into the acceptor Ti plasmid by a single cross over between homologous pBR322 sequences of the Ti plasmid and the intermediate vector. However, cointegrate vectors are very large because the T-DNA region and the *vir* genes are on the same plasmid. On the other hand, the binary vector strategy provides the manipulated disarmed T-DNA and the *vir* functions on separate plasmids. Therefore the T-DNA plasmid is small enough to have unique restriction sites and to be manipulated using standard techniques.

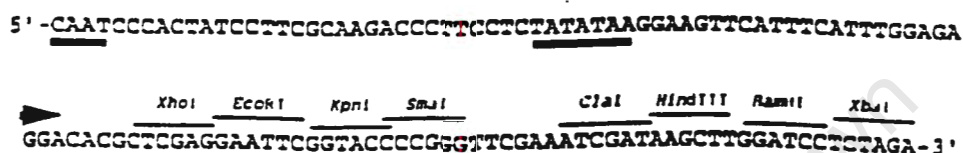
Binary vectors contain origins of replication and selectable markers functional in *E. coli* and *A. tumefaciens*. These vectors contain 25-bp border repeats between which are located appropriate cloning sites for insertion of foreign DNA and selectable markers functional in plant cells. This plasmid is introduced into an *A. tumefaciens* strain which harbours a Ti-plasmid that contains the *vir* region but no T-DNA. In this manner, the *vir* functions are supplied in *trans*, allowing for transfer of the recombinant T-DNA into the plant genome.

A binary vector system was used to introduce the *P. fluorescens* strain 17 ACC deaminase gene into *A. tumefaciens*. This system makes use of two vectors. The gene of interest is cloned into the primary cloning vector, pART7 (Figure 4.1A), to form part of an expression cartridge. The entire expression cartridge is then removed from pART7 and cloned into the secondary vector, pART27 (Figure 4.1C), which contains the essential elements for T-DNA transfer. The absence of any AUG translational initiation signals in the multiple cloning site (mcs) (Figure 4.1B) ensures that translation will begin at the first AUG of the cloned DNA (Gleave 1992). The entire expression cartridge is flanked by *NotI* sites which facilitate its introduction into pART27. The occurrence of the 8 bp *NotI* recognition sequence is rare in most genes (Gleave 1992). The *lac Z'* gene of pART27 possesses a unique *NotI* site which allows blue/white screening of recombinants in *E. coli*.

A



B



C

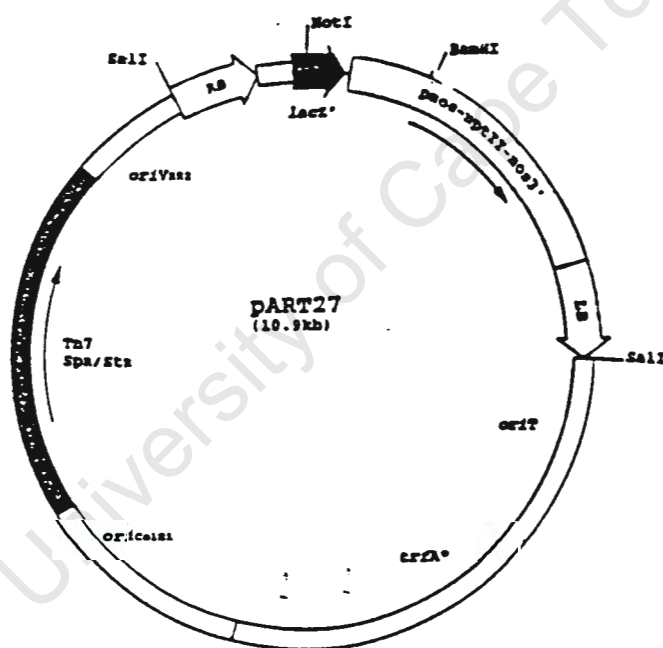


Fig. 4.1 pART binary vector system

(A) Primary cloning vector, pART7. The CaMV 35S promoter is represented by the open box with the arrow indicating the direction of transcription. The multiple cloning site is indicated and the MCS 3' region is represented by the open box.

(B) Nucleotide sequence of the transcriptional elements of the CaMV 35S promoter and the multiple cloning site of pART7. The TATA box and CAAT box of the CaMV 35S promoter are underlined and the arrow represents the transcriptional start point. The unique restriction sites of the multiple cloning site are indicated.

(C) Binary vector, pART27. The right border (RB) and left border (LB) are indicated by the arrowed boxes. The lacZ' region is represented by the dark arrowed box. The chimeric *nptII* region and direction of transcription is indicated.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains and plasmids

The plasmids pART7 and pART27 were obtained from A. Gleave (Gleave 1992). They and their recombinants were maintained in *E. coli* DH5 α cells (Hanahan 1983). Binary vector derivatives were transformed into *A. tumefaciens* C58C1 cells (Koncz and Schell 1986), which possessed a resident disarmed Ti-plasmid, pMP90, which supplied the Vir proteins in *trans*. Cells containing pART7 derivatives were stored on LB agar containing ampicillin (100 μ g/ml), and cells possessing pART27 recombinants were grown on media with either spectinomycin (100 μ g/ml), streptomycin (25 μ g/ml) or kanamycin (20 μ g/ml), or a combination of these antibiotics. *A. tumefaciens* C58C1 cells were maintained on LB agar containing rifampicin (100 μ g/ml).

4.3.2 PCR amplification of the *P. fluorescens* strain 17 ACC deaminase gene

Forward and reverse PCR primers were designed to include the start and stop codons of the *P. fluorescens* strain 17 ACC deaminase gene respectively. The forward primer had an *Eco*R1 site at its 5' end, and the reverse primer a *Cla*I site at its 5' end. PCR amplification reactions contained 0.125 μ M of each primer and 1 ng of template DNA (pBP1 carrying the ACC deaminase gene). DMSO (10%), 200 μ M each nucleotide and 100 mM MgCl₂ were also included in reactions and the final reaction volume was 50 μ l. Promega Taq DNA polymerase and buffer were used.

Reaction mixtures were subjected to 30 cycles of amplification. Each cycle had a denaturation step of 93°C (60 sec), annealing step of 60°C (30 sec) and an extension step of 72°C (60 sec). A final extension reaction of 72°C for 5 min was included. After amplification, samples were electrophoresed through a 0.8% agarose gel and the 1034-bp PCR fragment containing the ACC deaminase gene was eluted using the GeneClean^R procedure.

4.3.3 Cloning the ACC deaminase gene, analysis of recombinants, and transfer into *A. tumefaciens*

The 1034-bp PCR product (1 μ g) was digested with 1 unit of each of the restriction endonucleases *EcoR*I and *Cla*I for 2 hours at 37°C. This fragment was ligated into pART7 which had been digested with the same enzymes. pART7 recombinants transformed into DH5 α were patched onto M9 minimal medium containing ACC as the sole nitrogen source. Clones showing positive growth on this medium were subjected to PCR amplification of the ACC deaminase gene (Section 4.3.2). A positive pART7 recombinant was digested with *Not*I to release the expression cartridge. The sample was run on a 0.7% agarose gel, and the relevant DNA band purified using the GeneClean^R procedure. It was cloned into *Not* I-digested pART27 and treated with calf intestinal phosphatase (Appendix A) to prevent vector religation. Several positive white DH5 α transformants were selected, and tested for the presence of the expression cartridge by digestion with *Bam*HI, and PCR analysis (Section 4.3.2).

Transformation of *A. tumefaciens* C58C1 (pMP90) with the pART27 recombinants was carried out using the freeze thaw method as outlined by Draper *et al.* (1988). Confirmation of transformation was carried out by PCR analysis of selected colonies.

4.4 RESULTS AND DISCUSSION

4.4.1 PCR amplification of *P. fluorescens* strain 17 ACC deaminase gene

Sequencing the *P. fluorescens* strain 17 ACC deaminase gene showed that there was an out-of-frame ATG codon 26 bp upstream from the ATG start codon which codes for the first amino acid, methionine (Figure 4.2). This proved to be problematic when subcloning this gene into a plant vector. As there were no unique restriction enzyme sites between the two ATG codons, PCR primers were designed so that the ACC deaminase gene could be amplified, excluding the out-of-frame ATG codon.

The forward primer was identical to sequence 69 to 85 of the *P. fluorescens* ACC deaminase gene and had an *EcoRI* site plus 1 extra base at the 5' end (Figure 4.3). The last 3 bases included the ATG initiation codon. The reverse primer was complementary to the sequence from 1096 to 1110 of the ACC deaminase gene possessed bases complementary to the TGA stop codon as well as a *ClaI* site and two extra nucleotides at its 5' end. The enzymes *EcoRI* and *ClaI* do not cut the ACC deaminase gene internally.

- A. 5'-GGAATTCAAGGAGCAGAGTCATG-3'

- B. 5'-GGATCGATATTGGCGTTAATCAG-3'
 =====

Figure 4.3. Oligonucleotide primers used for amplification of the *P. fluorescens* strain 17 ACC deaminase gene.

- A. Forward PCR primer. The *EcoRI* site is represented by a single underline.
B. Reverse primer. The *ClaI* site is represented by a double underline.

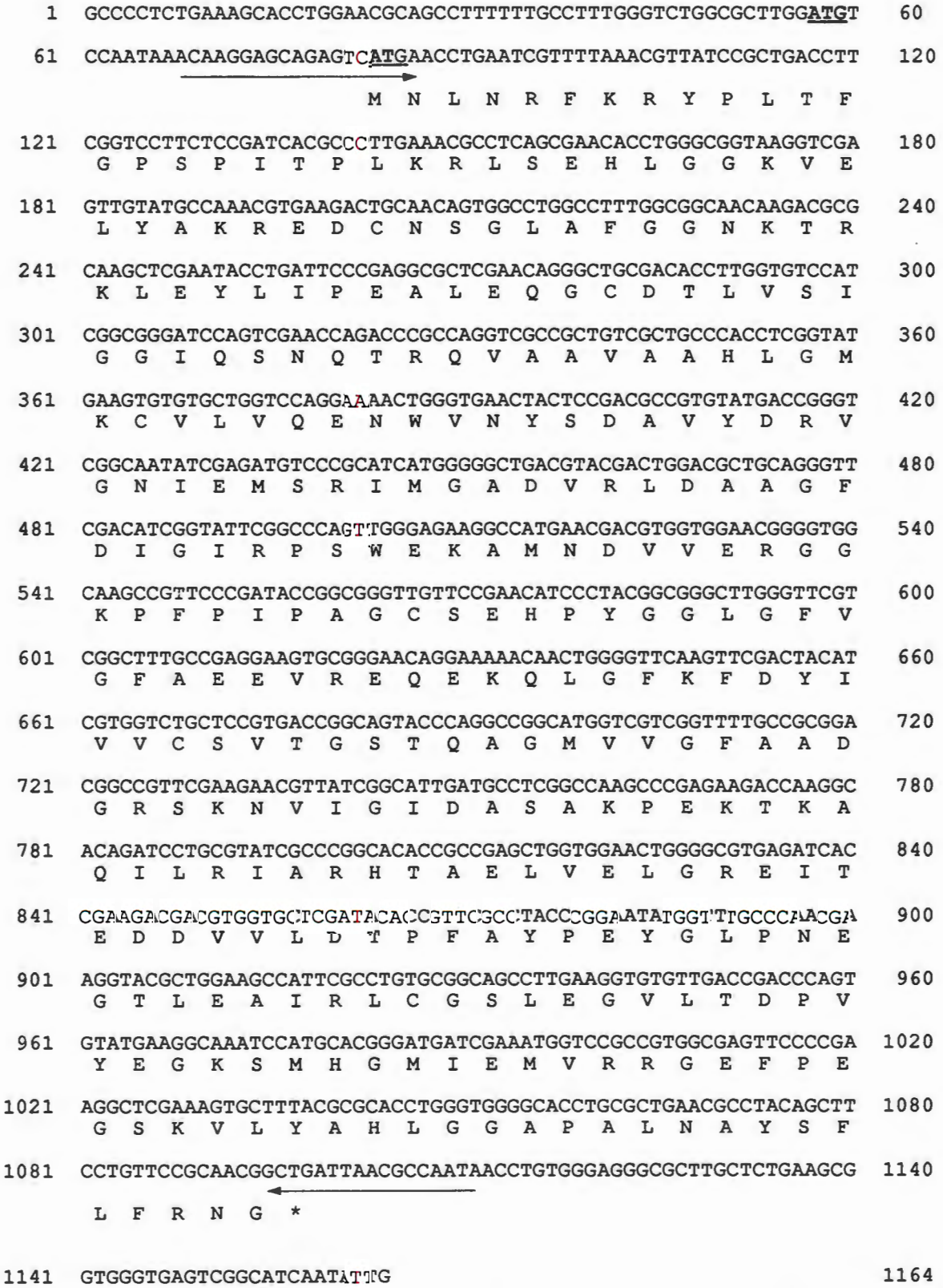


Fig 4.2 *P. fluorescens* strain 17 ACC deaminase gene sequence showing PCR primer binding sites.

The forward and reverse primer binding sites are shown by arrows. ATG codons are underlined.

4.4.2 Cloning the ACC deaminase PCR product into pART7

The ACC deaminase PCR product (Figure 4.4) was cut with *EcoRI* and *ClaI*, ligated into pART27 and transformed into *E. coli* DH5 α . Three hundred transformants were patched onto ACC-M9 minimal medium. Although the CaMV 35S promoter is a viral promoter that works optimally in plant cells, it does allow inefficient transcription in prokaryote systems (A. Hackland, personal communication).

253 colonies showed slight growth on the plates, and one colony displayed exceptional ACC degrading ability. PCR screening for the ACC deaminase gene indicated that all the tested colonies possessed the gene (Figure 4.5). This was unexpected as the colonies showed varying growth rates on ACC-containing medium. Restriction analysis with *BamHI* which gives band sizes of 804 and 5138 bp (see Figure 4.7) indicated that the colony displaying rapid growth carried a plasmid with a deletion as it was slightly smaller than the plasmids of slow growing colonies (Figure 4.6). Digestion with *NotI* showed that the expression cassette was released from pACC1 and pACC102 as expected, but not from p65M (the plasmid from the fast growing colony) which was only cut once. This suggests that the deletion occurred either at the start of the CaMV 35S promoter, or in the 3' region of the *ocs-3'* sequence. Since polyadenylation signals (coded for by the *ocs-3'* region) play no recognisable role in the processing of prokaryotic mRNA transcripts (Freifelder 1987) the deletion, which led to increased expression in *E. coli*, is more likely to have occurred in the 35S promoter.

4.4.3 Cloning the pACC1 expression cassette into pART27

As p65M had lost a *NotI* site, the 3017-bp cassette was cut out of pACC1 with *NotI* and cloned into the *lac Z'* gene of pART27. Several hundred white colonies were obtained when DH5 α transformants were plated on X-gal. Only 11 blue colonies grew indicating that the alkaline phosphatase treatment had been effective. Restriction digests with *BamHI* showed that the expression cartridge had been cloned into the *NotI* site of pART27 in both directions (results not shown). Maps of these plasmids are shown in Figure 4.7. The ACC deaminase gene of pAR1 is transcribed in the same direction as the *nptII* gene whereas this gene is transcribed in the opposite

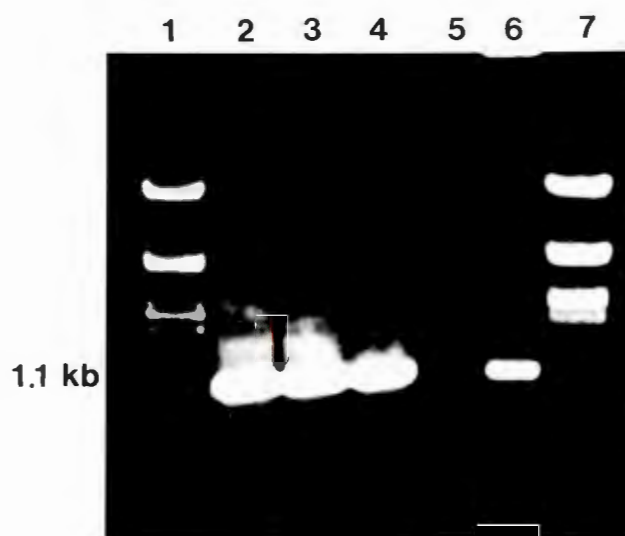


Fig. 4.4 PCR amplification of the *P. fluorescens* strain 17 ACC deaminase gene.

Lanes 1 and 7, molecular size DNA, λ DNA digested with *Pst*I; lanes 2, 3, 4 and 6, ACC deaminase gene products amplified from pBP1, pSN2, pBC17 and strain 17 respectively. Lane 5 shows the PCR reaction of pSK, a negative control.

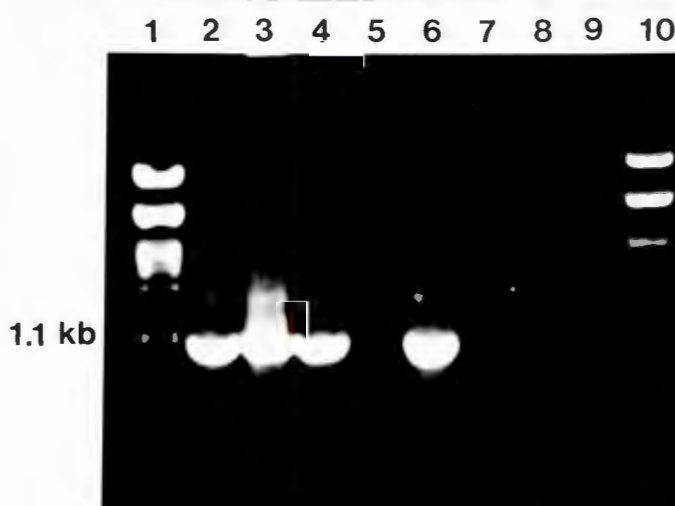


Fig. 4.5 PCR analysis of pART7 recombinants.

Primers were used to amplify the ACC deaminase gene. Lanes 1 and 10, molecular size markers, λ DNA digested with *Pst*I; lanes 2-9, p65M, pACC1, pBP1 (positive control), pSK (negative control), pACC102, pART7 (negative control), pART27 (negative control), and the no DNA control respectively.

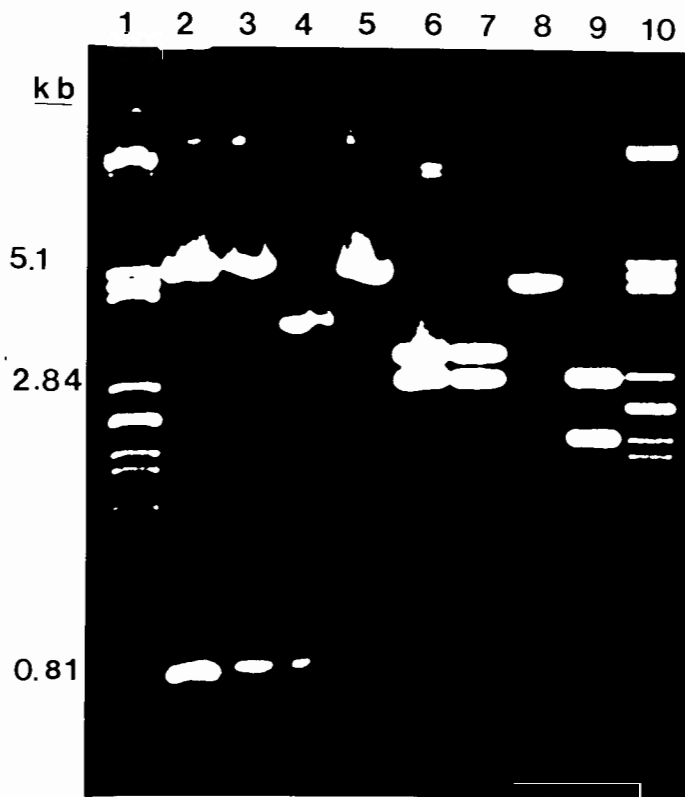
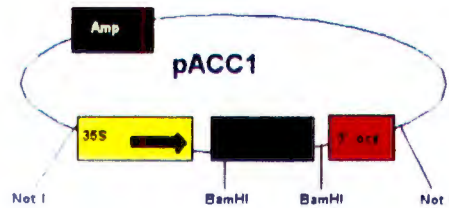


Fig. 4.6 Digestion of pART7 recombinants with *Bam*HI and *Not*I.

Lanes 1 and 10, molecular size markers, λ DNA digested with *Pst*I; lanes 2-5, pACC1, pACC102, p65M and pART7 digested with *Bam*HI respectively; lanes 6-9, pACC1, pACC102, p65M and pART7 digested with *Not*I respectively. p65M was isolated from a colony which grew well on minimal medium plates with ACC as the sole nitrogen source, whereas pACC1 and pACC102 were isolated from slow growing colonies. p65M appears to have a deletion in the 5' region of the CaMV 35S promoter.

A.



B.

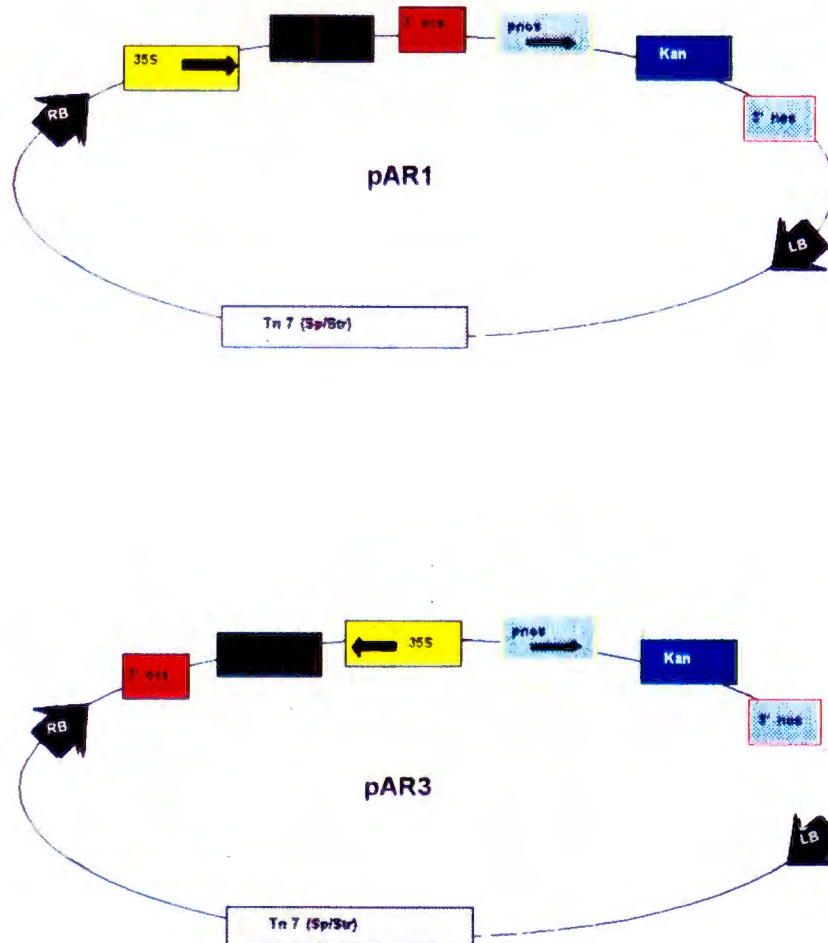


Fig. 4.7 Schematic representation of pART7 and pART27 derivatives.

(A) pACC1, pART7 containing the ACC deaminase gene. The direction of transcription of the gene (ACC) from the CaMV 35S is shown, as is the *ocs-3'* termination sequence.

(B) pAR1 and pAR3, pART27 derivatives containing the ACC deaminase expression cassette of pACC1. The right border and the left border are indicated by arrowed boxes. The chimeric *npIII* region (*pnos-Kan-nos3'*) and direction of transcription are shown.

direction in pAR3. It was decided to transfer both of these constructs to plant cells to see if there was any difference in the level of ACC deaminase expression.

PCR amplification of these *E. coli* transformants revealed that both pAR1 and pAR3 contained the 1034-bp ACC deaminase gene band (Figure 4.8). The transfer of these plasmids into *A. tumefaciens* C58C1 was very inefficient but PCR screening for the presence of the ACC deaminase gene indicated a positive result (Figure 4.9). These strains are now ready for transfer into pome and stone fruit plants to generate transgenic fruit with potentially delayed ripening and thus a longer shelf life.

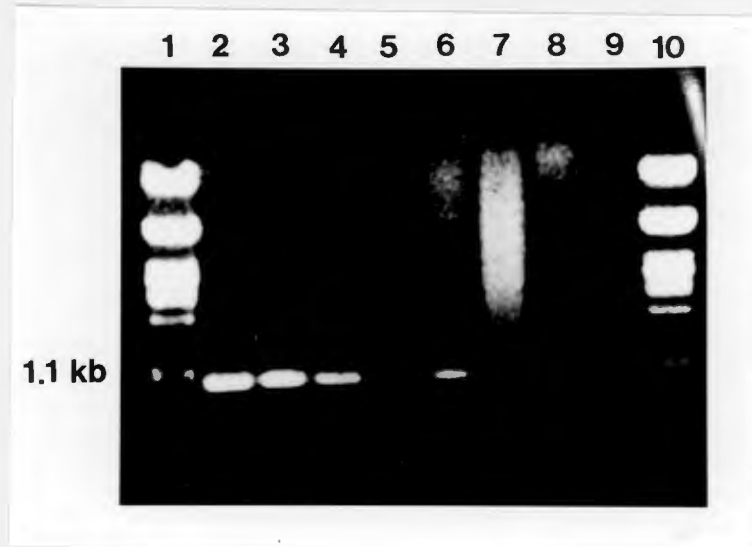


Fig. 4.8 PCR analysis of *E. coli* cells harbouring pART27 recombinants.

Lanes 1 and 10, molecular size markers, λ DNA digested with *Pst*I. Lanes 2-9 represent PCR products from pAR1, pAR2, pAR3, pACC102, pACC1, pART7, pART27 and the no DNA control respectively.

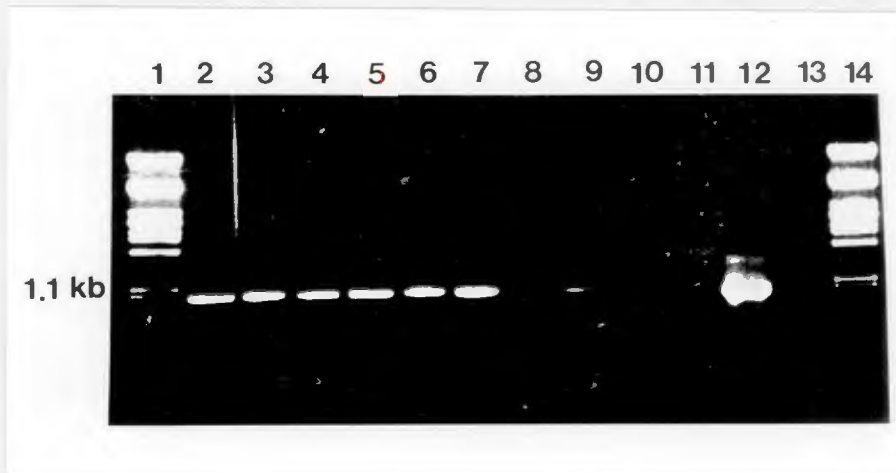


Fig. 4.9 PCR analysis of *A. tumefaciens* cells harbouring pART27 derivatives.

Lanes 1 and 10, DNA molecular size markers, λ DNA digested with *Pst*I. Other lanes represent PCR products of tested colonies. Lanes 2-5, colonies transformed with pAR1; lanes 6-9, colonies transformed with pAR3; lane 10, pART27 in *A. tumefaciens* (negative control); lane 11, pART27 in *E. coli*; lane 12, pAR1 in *E. coli* (positive control); lane 13, no DNA control.

4.5 CONCLUSION AND WORK FORTHCOMING FROM THIS PROJECT

The introduction of the ACC deaminase-containing plasmids, pAR1 and pAR3, into *A. tumefaciens* C58C1 could allow for the development of climacteric fruit species with delayed fruit ripening. The strains have been given to various ARC institutes for the development of transgenic plants. The Vegetable and Ornamental Plant Institute (VOPI) intend making tomato plants transgenic for the ACC deaminase gene. If this gene proves to be operative in tomatoes, Infruitec will introduce the construct into stone and pome fruits. Tomato plants are ideal for testing the effectiveness of ACC deaminase genes as they have relatively short regeneration times compared to the woody stone and pome fruit trees.

Because the *P. fluorescens* strain 17 ACC deaminase gene is 86% homologous to that of *Pseudomonas* sp. strain 65G, there is a high probability that it will delay the fruit ripening process. Klee *et al.* (1991) introduced the *Pseudomonas* sp. strain 65G ACC deaminase gene into tomatoes and demonstrated that the mature fruits remained firm for at least six weeks longer than the non-transgenic control fruit. These results thus indicated that ACC deaminase is useful for extending the shelf life of fruits and vegetables whose ripening is mediated by ethylene.

Future work will involve cloning the ACC deaminase gene in downstream of a fruit specific promoter. The phytohormone ethylene plays a role in a number of plant functions such as abscission of leaves, fruits and flowers, seed germination, and is stimulated in response to environmental stress such as mechanical wounding, waterlogging and pathogen attack (Raven *et al.* 1986). It may therefore be disadvantageous for the plant to have all its ethylene synthesis inhibited. Therefore cloning the ACC deaminase gene in front of fruit specific promoters such as the polygalacturonase gene promoter (Bird *et al.* 1988) or the 2A11 gene promoter (Van Haaren and Houck 1991) would delay ethylene biosynthesis in ripening fruit. However, care needs to be taken to ensure that the promoter of choice is active in a number of plant species and not just the species of origin. To date only tomato fruit

specific promoters have been characterised. Studies now need to be undertaken to identify promoters in other climacteric fruits and their subsequent host range.

Other work arising from the findings of this project will involve sequencing the pSK subclone pBG1 which contains the novel *P. fluorescens* strain 15 ACC deaminase gene. It would be interesting to observe whether this gene delays fruit ripening in the same manner, and to the same extent as the other ACC deaminase genes. Similarly isolation and characterisation of yeast ACC deaminase genes could prove to be useful in comparative ACC deaminase studies as yeasts belong to a different kingdom from bacteria. However before this is possible, ACC deaminase extraction and assay techniques need to be optimised for yeasts.

In conclusion, introducing a bacterial ACC deaminase gene into plants gives rise to the potential for delaying fruit ripening, thus preventing fruit spoilage. This could save billions of dollars every year, as farmers would no longer have to apply inhibitors of ethylene synthesis, or store fruit under refrigerated conditions or hypobaric pressures. Furthermore ACC deaminase genes have an advantage over antisense ACC synthase and ACC oxidase genes in that they are not specific for a particular plant species. This allows one to introduce this gene into a number of different plant species to delay fruit ripening thus cutting the costs of producing either ACC synthase or ACC oxidase antisense genes for each plant species.

APPENDIX A

Methods

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APPENDIX A

A1. Preparation of *Pseudomonas* sp. genomic DNA

Genomic DNA was prepared according to the method of Ausubel *et al.* (1989).

1. A 100 ml culture was grown to saturation at 30°C with shaking.
2. Bacterial cells were collected by centrifuging samples for 5 min at 5000 rpm.
3. Cells were resuspended in 9.5 ml TE buffer. Cell lysis was performed by adding 500 μ l of 10% SDS and 50 μ l of 20 mg/ml proteinase K and incubating the sample for 1 hr at 37°C.
4. 1.8 ml of NaCl was added and the sample was mixed thoroughly before the addition of 1.5 ml CTAB/NaCl solution.
5. After an incubation of 20 min at 65°C, an equal volume of chloroform/isoamyl alcohol was added, and the sample was centrifuged at 7000 rpm for 5 min to separate the phases.
6. The aqueous supernatant was transferred to a fresh tube using a wide-bored pipette, and the DNA was precipitated out of solution with 0.6 volumes of isopropanol.
7. The DNA was pelleted at 10000 rpm for 10 min, and washed with 70% ethanol.
8. The pellet was resuspended in 500 μ l TE, pH8 and the concentration of DNA was determined spectrophotometrically by measuring the A_{260} between 210 nm and 310 nm of a 1 in a 100 dilution. The relationship of $A_{260} = 1$ for 50 μ g/ml DNA was used.

Preparation of DNA for genomic library construction was done according to the method of Klee *et al.* (1991).

1. A 200 ml *Pseudomonas* sp. culture was grown in LB broth overnight at 37°C.
2. The cells were collected and resuspended in 10 ml of 25 mM Tris-HCl, pH8, 10 mM EDTA. SDS was added to a final concentration of 1%, and the suspension was subjected to three freeze-thaw cycles each consisting of immersion in dry ice for 15 min and in water at 70°C for 10 min.

3. The lysate was extracted four times with equal volumes of phenol:chloroform, and was precipitated with 2.5 volumes ethanol and one tenth volume of 4 M lithium chloride.
4. The pellet was resuspended in 5 ml TE, pH 7.5, and dialysed for 16 h at 4°C against 2 l of the same buffer. The concentration of DNA was determined spectrophotometrically as above.

A2. Preparation of yeast genomic DNA.

Yeast genomic DNA was prepared according to Sanglard (1993).

1. LB broth (5 ml) was inoculated and the culture was grown up overnight at 30°C.
2. Yeast cells were pelleted by centrifugation for 5 min at 5000g, washed twice with TE, pH 8, and protoplasted in 2 ml PRO buffer with 0.75 mg Amylase 100T and 15 mM β -mercaptoethanol.
3. After 30 min incubation at 37°C, the protoplasts were washed twice by centrifugation with the same buffer.
4. Protoplasts were resuspended in 2 ml lysis buffer and incubated at 65°C for 30 min.
5. Proteins were removed from the solution by the addition of one fifth volume 4 M potassium acetate, pH 5, and incubation at 4°C for 10 min.
6. After centrifugation for 5 min at 6000g, the DNA in the supernatant was precipitated with two volumes of ethanol. The pellet was washed with 70% ethanol and resuspended in 0.5 ml TE, pH 8, and treated with 20 μ g RNase for 15 min at 37°C.
7. The DNA was reprecipitated with one volume of isopropanol, washed with ethanol and resuspended in TE, pH 8.
8. DNA concentration was determined spectrophotometrically as described in A1.

A.3 Large scale isolation of plasmid DNA

DNA isolations were performed according to Ish-Horowicz and Burke (1981).

1. A 200 ml culture was grown overnight with shaking at 37°C in the presence of 100 μ l/mg ampicillin.
2. The cells were harvested in a GSA centrifuge tube at 5000 rpm for 5 min.
3. The pellet was resuspended in 4 ml of Solution I (50mM glucose, 25mM Tris-HCl, pH 8.0), transferred to a SS34 centrifuge tube and left for 5 min at room temperature.
4. 8 ml of Solution III (0.2 M NaOH, 1% (w/v) SDS) was added. The tube was rolled gently to mix the solutions and was incubated on ice for 5 min.
5. 6 ml of Solution III (5M potassium acetate, pH 4.8) was added, the tube was mixed well and incubated for 5 min on ice.
6. The debris was spun down at 15 000 rpm for 15 min and the supernatant decanted into a new SS34 tube.
7. An equal volume of isopropanol was added and the tube was centrifuged at 15 000 rpm for 15 min.
8. The pellet was washed with 70% ethanol, the tube was inverted to drain it and the pellet was resuspended in 4.2 ml Tris-EDTA (TE) buffer, pH 8.0. 4.4 g of cesium chloride (CsCl) was added and dissolved and 400 μ l of ethidium bromide (EtBr) (10 mg/ml) was added.
9. The tube was centrifuged at 15 000 rpm for 15 min to precipitate any remaining protein debris.
10. The refractive index of the solution was adjusted to 1.394.
11. The sample was sealed in a Beckman Quickseal ultracentrifuge tube (5ml) and centrifuged for a minimum of 6 h at 55 000 rpm in a Beckman Vti 65 rotor at 15°C.
12. The plasmid band was extracted in the smallest possible volume from the side of the tube using a 1 ml syringe and a 1.2 mm gauge needle under long wave length UV light (350 nm).
13. The EtBr was extracted at least three times using equal volumes of salt saturated isopropanol.
14. Two volumes of water were added to the DNA solution. One volume of

isopropanol was added to this, and DNA was pelleted at 12 000g in a microfuge for 10 minutes at room temperature.

15. The pellet was washed with 1 ml of 70% ethanol at 12000g for 10 min.
16. The DNA was resuspended in 200 μ l TE, pH 8 and the concentration of DNA was determined spectrophotometrically as described in A1.

A4. Small scale isolation of plasmid DNA (miniprep)

The method is a scaled down version of the above maxiprep method.

1. LB broth (700 μ l) containing 100 ug/ml ampicillin was pipetted into Eppendorf microcentrifuge tubes which were then inoculated and grown at 37°C with vigorous shaking overnight.
2. Cells were harvested by centrifugation at 10 000g for 1 min.
3. Solution I (150 μ l) was added and the tube was vortexed until the pellet was resuspended.
4. Solution II (300 μ l) was added, the tube inverted and incubated for 5 min on ice.
5. Solution III (225 μ l) was added and the tube was inverted gently a few times. After 5 min on ice, the cellular debris was collected by centrifugation at 10 000g for 5 min.
6. The supernatant was removed to a new Eppendorf tube. One volume of isopropanol was added, mixed and spun at 10 000g for 15 min.
7. The DNA pellet was washed with 70% ethanol, and resuspended in TE, pH 8 containing 10 ug/ml ribonuclease A (RNase).

A5. Restriction endonuclease digestion of DNA

Restriction digests were performed as outlined by Sambrook *et al.* (1989). Restriction digests of miniprep DNA typically contained 10 μ l of DNA, 1 unit of endonuclease and 2 μ l of the appropriate restriction endonuclease buffer in a total volume of 20 μ l. Restriction digests of maxiprep DNA were performed in 20 μ l and contained 1 unit of endonuclease for every microgram of plasmid and 2 μ l of the appropriate restriction endonuclease buffer. All digests were left at 37°C for 2 hr unless stated otherwise by the manufacturer.

A6. Alkaline phosphatase treatment of plasmid DNA

Alkaline phosphatase treatment of plasmid DNA was performed according to Sambrook *et al.* (1989).

1. Restriction endonucleases in typical 20 μ l plasmid digests were inactivated by heating the sample for 10 min at 65°C.
2. The sample was made up to 100 μ l with distilled water and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and the tube was shaken well.
3. The Eppendorf tube was centrifuged for 5 min at 5000g. The upper aqueous phase was removed, and any remaining phenol was extracted from this phase with one volume of chloroform:isoamylalcohol (24:1).
4. DNA was precipitated from the aqueous phase with two and a half volumes of 100% ethanol and one tenth volume of 4M LiCl.
5. The DNA was pelleted and washed with 70% ethanol before being resuspended in 44 μ l of distilled water.
6. Calf intestinal alkaline phosphatase (2 μ l) and 5 μ l of the appropriate buffer was added to the DNA, and the solution was incubated for one hour at 37°C.
7. The reaction was stopped by heating the sample to 65°C for 10 min.
8. DNA was precipitated from the solution as outlined in step 4.

A7. DNA ligation reactions

Ligation reactions were allowed to proceed overnight at 16°C. Ligation reaction volumes were usually 20 μ l containing 2 μ l of ligation buffer with ATP (Boehringer Mannheim) and 1 unit of T4 ligase.

A8. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using horizontal submerged gels (Sambrook *et al.* 1989). Agarose was dissolved in either Tris-acetate EDTA (TAE) or Tris-borate EDTA (TBE) buffer to a final concentration of 0.7, 0.8 or 1%. Electrophoresis loading buffer, containing the dye bromophenol blue, was added to the DNA samples (one sixth the final volume) before they were loaded into the wells of agarose gels. Long gels were run at 100 V constant voltage for about 5 h or

overnight at 30 V. Minigels were run at 100 V constant voltage for 2 h. Gels contained 5 μ l of EtBr (10 mg/ml stock) in 100 ml of agarose so that DNA bands could be visualised using a 264 nm transilluminator. If DNA fragments were to be isolated from the agarose gel for subsequent ligation reactions, the 310 nm transilluminator was used for only a few seconds. Gel photography was carried out using a Polaroid CU-5 Land camera fitted with a red filter. Polaroid type 667 film (ASA 3000) was used with an exposure time of 1-2 sec at f4.7.

A9. Purification of DNA fragments

The Geneclean^R Kit (Bio 101) was used according to the manufacturer's instructions to isolate DNA fragments from agarose gel slices.

1. The appropriate agarose gel slice (0.8% agarose) was cut out of the gel under long wave UV light (310 nm).
2. 2.5 volumes of saturated sodium iodide solution were added to the gel slice. The tube was left at 50°C for 5 min.
3. Glassmilk (5 μ l) was added and the solution was mixed well and left on ice for 5 min.
4. The sample was spun for 5 s to pellet the glassmilk and the supernatant was discarded.
5. The pellet was washed three times with 500 μ l ice cold NEW buffer.
6. The solution was spun as before. The DNA was eluted from the glassmilk by dissolving the pellet in 10 μ l TE, pH8.

A10. Alkali DNA blotting

1. The DNA fragments to be blotted were separated by 0.8% agarose gel electrophoresis in TBE buffer.
2. The DNA was depurinated by soaking the gel in 0.25 M hydrochloric acid (HCl) for 15 min with gentle shaking. The gel was briefly rinsed in distilled water.
3. A capillary transfer system was set up. A Whatmann 3MM filter wick was wet with 0.4 M NaOH and placed over a glass bridge so that the wick ends were

touching the bottom of a reservoir tank containing 0.4 M NaOH. The gel was placed on the wick, on top of which was placed a wetted nylon Hybond N+ membrane and three sheets of Whatmann 3MM filter paper. All air bubbles were removed before placing a stack of absorbent pads ten centimetres in height on top of the membrane. A one kilogram weight was used to compress the pads. DNA transfer from the gel to the nylon membrane took place via capillary action for 20 h with periodic replacement of the absorbent pads.

4. The membrane was dried at 37°C for 1 h. The membrane was then ready for prehybridisation.

A11. Southern hybridisation of DNA to a DIG-labelled probe

Prehybridisation, hybridisation and detection procedures were carried out as according to the instructions of the manufacturer of the Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim).

A11.1 Hybridisation

1. The membrane was allowed to prehybridise for a minimum of 4 h in prehybridisation buffer in a plastic container at 65°C with gentle shaking.
2. The DIG-labelled DNA probe (25 ng DNA/ml buffer) was heated in a boiling water bath for 10 min to denature the DNA, and then immediately chilled on ice.
3. The membrane was placed in a hybridisation bag which had been sealed on three sides. Hybridisation buffer (20 ml) was added and the bag was sealed making sure that all the air bubbles had been removed. Hybridisation was allowed to proceed overnight at 65°C.
4. The probe was poured into a capped plastic tube and was frozen at -20°C for further use.
5. The membrane was given two 5 min washes in 2X SSC/0.1% SDS at room temperature, and was then washed twice for 15 min at 65°C in 0.1X SSC/0.1% SDS.

A11.2 Chemiluminescent detection

All volumes are for a membrane size of 100 cm²

1. After hybridisation and post-hybridisation washes, the membrane was equilibrated in wash buffer for approximately 5 min.
2. The membrane was incubated in buffer 2 for 30 min with gentle agitation.
3. Anti-DIG-alkaline phosphatase fragments were diluted 1:100000 in buffer 2. The membrane was incubated with 20 ml of this solution for 30 min.
4. The antibody solution was discarded, and unbound conjugate was removed by 2 X 15 min washes with 100 ml wash buffer.
5. The membrane was equilibrated for 5 min in buffer 3.
6. The AMPPD^R stock solution (10 mg/ml) was diluted 1:200 in buffer 3. The membrane was incubated with the lumigen for 5 min with gentle agitation.
7. The damp membrane was sealed in a hybridisation bag and was exposed to XAR-5 autoradiographic film for 10-30 min.

A12. Exonuclease III shortening of insert DNA

1. Plasmid DNA (12ug) was double digested to completion with the appropriate restriction endonucleases generating a 3' overhang which is susceptible to the exonuclease III enzyme, and a 5' overhang adjacent to vector sequence which is resistant to the enzyme.
2. The DNA was precipitated by adding one tenth volume of 5 M sodium perchlorate and an equal volume of isopropanol. The pelleted DNA was resuspended in 100 µl of exonuclease III buffer.
3. Eleven Eppendorf tubes each containing 12 µl of freshly prepared S1 mix were placed on ice.
4. The tube containing the DNA mixture was equilibrated at 37°C, and at T=0 a 4.5 µl sample was removed and placed in the first of the 11 tubes as an undigested control sample.
5. The shortening reaction was started by the addition of 150 U of exonuclease III to the DNA tube.
6. While at 37°C, samples (4.5 µl) were removed from the DNA tube at 30 s intervals and were added to the S1 tubes. Solutions were mixed well by

vigorous pipetting.

7. The Eppendorf tubes containing S1 mixes were raised to room temperature and incubated for 30 min.
8. The action of S1 nuclease was inhibited by the addition of 1.8 μ l of S1 stop. Tubes were placed at 70°C for 10 min.
9. To confirm that shortening reactions were successful, 4 μ l from every second tube was run on a 0.8% agarose gel.
10. Klenow mix (1.8 μ l) and 1 μ l of klenow enzyme were added to each tube and left for 5 mins at room temperature to blunt the ends of the DNA fragments.
11. dNTPs (1 μ l of 0.125 mM solution) were added to each tube and left for 15 min at room temperature.
12. Half of the sample from each tube was added to 60 μ l of ligation mix (ligation buffer, T4 ligase and sterile water). Ligations were carried out overnight at 16°C. Competent *E. coli* cells were transformed. Minipreparation and digestion of plasmid DNA was performed to find the appropriate shortened plasmids.

A13. Nucleotide sequencing

Nucleotide sequencing was according to the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977). The Sequenase^R Version 2.0 DNA Sequencing Kit was used according to the manufacturer's instructions. This kit uses the T7 DNA polymerase (Sequenase^R) enzyme.

A13.1 Primer annealing reaction

1. 10 μ g of freshly maxiprep DNA resuspended in TE, pH 8, was diluted to 18 μ l with sterile distilled water.
2. 2 N NaOH (2 μ l) was added and the tube was incubated at 37°C for 30 min to denature the double stranded DNA.
3. The tube was placed on ice immediately and 4 μ l 3 M sodium acetate and 150 μ l absolute ethanol was added.
4. The tube was placed at -70°C for 15 min and then microcentrifuged for 15 min at 4°C.

5. 70% ethanol (500 μ l) was added and the tube was centrifuged again so that the pellet was spun through the ethanol wash. The supernatant was carefully discarded.
6. The DNA pellet was resuspended in 7 μ l sterile distilled water to which 2 μ l of reaction buffer and 1 μ l primer (2 pmol) was added.
7. The tubes were incubated at 65°C for 10 min and then cooled to 37°C.

A13.2 Sequencing reactions

1. Termination tubes were prepared as follows: 1.5 μ l extension mix and 1 μ l dideoxy-termination mix was added to G and C termination tubes. A and T termination tubes contained 1 μ l extension mix and 1.5 μ l dideoxy-termination mix. If more than one template was sequenced, these volumes were scaled up and 2.5 μ l aliquots were added to each termination tube.
2. The labelling mix was prepared on ice and the amount per template was as follows:
 - 1.0 μ l DTT
 - 2.0 μ l Labelling mix minus dATP
 - 1.7 μ l DMSO
 - 0.5 μ l SSB
 - 2.0 μ l T7 polymerase
 - 0.5 μ l ^{35}S -dATP
3. The template tubes were placed at room temperature and 8 μ l of the prepared labelling mix was added to each tube. The labelling reaction was left for 20 min.
4. The termination tubes (A, C, G, T) were prewarmed in a heating block to 48°C for 30 s.
5. An aliquot of 3.6 μ l from each labelled template tube was added to each of the 4 prewarmed termination tubes. Termination reactions were left to proceed for 10 min at 48°C.
6. Klenow enzyme (1 μ l of 0.25 u solution) was placed in each termination and

tube and the incubation continued for 4 min.

7. Stop solution (5 μ l) and 1 μ l of proteinase K (0.1 μ g/ml) mixed into each tube. After 5 min, termination reactions were placed at 65°C for 20 min to inactivate the proteinase K.
8. Prior to electrophoresis, the samples were denatured at 95°C for 3 min and then placed on ice.
9. Aliquots (3–4 μ l) were run on 6% polyacrylamide gels with Tris-taurine-EDTA running buffer for 2 to 6 h at 80 Watts.
10. The gel was dried at 80°C onto Whatmann 3MM blotting paper using a Dual Temperature Slab Gel Dryer (Hoefer Scientific Instruments) for 30 min and then exposed to XAR-5 autoradiographic film for a minimum of 16 h.

A14. Preparation and transformation of competent *E. coli* cells

The method of Draper *et al.* (1989) was used for the transformation of *E. coli* cells.

1. A 1/100 dilution of an overnight culture was made into 100 ml of LB broth, and was grown with shaking at 37°C until the OD₆₀₀ reached 0.35.
2. The culture was transferred into a GSA bottle and chilled on ice for 15 min.
3. Cells were pelleted at 2500 rpm for 5 min at 4°C.
4. Cells were gently resuspended in 21 ml ice-cold TFB1, and incubated on ice for 90 min.
5. Samples were centrifuged at 2500 rpm for 5 min at 4°C. Cells were resuspended in 35 ml chilled TFB2. These cells were aliquoted (100 μ l) into 1.5 ml Eppendorf tubes. These cells were competent and were either used immediately for transformation or frozen in liquid nitrogen and stored at -70°C.
6. Plasmid DNA (10 ng) was added to 100 μ l of competent cells and incubated on ice for 20 min.
7. Cells were heat shocked by placing the tubes in a 37°C water bath for 60 s. Tubes were returned immediately to ice for a further 2 min.
8. LB broth (800 μ l) was added to each Eppendorf tube which was left to shake at 37°C for half an hour.
9. Cell aliquots (100 μ l) were plated onto LB plates containing 100 μ g/ml ampicillin or the relevant antibiotic.

A15. Preparation and transformation of competent *A. tumefaciens* cells

Competent *A. tumefaciens* cells were prepared according to the method of Draper *et al.* (1989).

1. *A. tumefaciens* containing an appropriate helper Ti-plasmid in 5 ml of LB broth overnight at 30°C.
2. The overnight culture (2 ml) was added to 50 ml LB broth in a 250 ml flask and left to grow to an OD₆₀₀ of 0.5 to 1.0.
3. The culture was chilled on ice before cells were collected at 5000 rpm for 5 min at 4°C.
4. The cells were resuspended in 1 ml of 20 mM ice-cold CaCl₂. Aliquots (0.1 ml) were dispensed into prechilled Eppendorf test tubes.
5. Plasmid DNA (1 ug) was added to the cells before they were frozen in liquid nitrogen.
6. Samples were thawed by incubating tubes in a 37°C water bath for 5 min.
7. LB broth (1 ml) was added to the tube and cells were incubated at 30°C for 4 h with gentle shaking.
8. Tubes were centrifuged for 30 s and resuspended in 0.1 ml LB broth.
9. The cells were spread on LB plates containing the relevant antibiotic. Transformed colonies were left to grow at 30°C for 3 days.

APPENDIX B

Buffers and solutions

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APPENDIX B

Standard buffers and solutions were prepared as outlined in Sambrook *et al.* (1989). Solutions were autoclaved at 121°C for 15 min or were filter sterilised through a 0.22 µm Millipore filter.

B1. Tris-EDTA (TE) buffer (pH 8.0)

Tris base	1.21 g
EDTA (0.5 M, pH 8.0)	2.0 ml
Distilled water to	1.0 l

The pH was adjusted to 8.0 with 0.1 M HCl

B2. 50 X Tris Acetate (TAE) buffer (pH8.0)

Tris base	242.0 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH8.0)	100.0 ml
Distilled water to	1 l

B3. 10 X Tris-Borate EDTA (TBE) buffer (pH 8.0)

Tris base	108.0 g
Boric acid	55.0 g
EDTA	20.0 ml
Distilled water to	1 l

B4. 10 X Tris-Taurine EDTA (TTE) buffer

Tris base	108.0 g
Taurine	36.0 g
EDTA	3.72 g
Distilled water to	1 l

B5. Gel electrophoresis loading buffer

Bromophenol blue	62.5 g
Sucrose	10.0 g
EDTA (0.5 M, pH 8)	1.0 ml
Distilled water to	25.0 ml

B6. Solutions for *Pseudomonas* sp. genomic DNA preparation**CTAB/NaCl solution**

NaCl	4.1 g
CTAB	10.0 g
(hexadecyltrimethyl ammonium bromide)	
Distilled water to	1 l

B7. Solutions for yeast genomic DNA isolation**PRO buffer**

1 M sorbitol
 25 mM EDTA
 20 mM Tris-HCl, pH 7.5

Lysis buffer

100 mM Tris-HCl, pH 8.0.
 50 mM EDTA
 0.5% SDS
 50 µg proteinase K

B8. Alkaline lysis buffers for preparation of plasmid DNA**Solution I**

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1 % SDS

Solution III

5 M potassium acetate 60.0 ml

Glacial acetic acid 11.5 ml

Distilled water to 28.5 ml

B9. Restriction enzyme buffers

Boehringer Mannheim buffers A, B, L, M and H were used with the appropriate restriction endonuclease.

Final concentration in mmol/l

<u>Stock solution</u>	A	B	L	M	H
Tris acetate	33	-	-	-	-
Tris-HCl	-	10	10	10	50
Mg-acetate	10	-	-	-	-
MgCl ₂	-	5	10	10	10
K-acetate	66	-	-	-	-
NaCl	-	100	-	50	100
Dithioerythritol	-	-	1	1	1
Dithiothreitol	0.5	-	-	-	-
2-mercaptoethanol	1	1	-	-	-
pH at 37°C	7.9	8.0	7.5	7.5	7.5

B10. DNA hybridisation solutions**20 X SSC**

NaCl	175.3 g
Trisodium citrate dihydrate	88.2 g
Distilled water	1 l
pH to 7.0	

Prehybridisation and hybridisation buffer

20 X SSC	50 ml
Skim milk powder	3 g
N-lauroylsarcosine, Na	0.2 g
SDS (0.02%)	400 μ l
Distilled water to	200 ml

B11. Chemiluminescent detection solutions**Buffer 1**

Maleic acid	23.2 g
NaCl	17.6 g

Adjust pH to 7.5 with NaOH pellets, make volume up to a litre and autoclave.

Wash buffer

Buffer 1	997 ml
Tween 20	3 ml

Block buffer

1% skim milk powder in buffer 1.

Buffer 3

1 M Tris-Cl (pH 8)	50 ml
5 M NaCl	10 ml

1 M MgCl_2	25 ml
Distilled water to	500 ml

B12. Exonuclease III shortening solutions

10 X S1 buffer

3 M potassium acetate	1.1 ml
5 M sodium chloride	5.0 ml
Glycerol	5.0 ml
ZnSO_4	30 mg
Autoclave	

Exo III buffer

1 M Tris-Cl, pH 8.0	660 μl
100 mM MgCl_2	66.4 μl
Distilled water	9.27 ml
Autoclave	

S1 mix

10 X S1 buffer	41 μl
Sterile water	258 μl
S1 nuclease	60 U

S1 stop

0.3 M Tris base

0.05 M EDTA

Autoclave but do not pH since high pH is needed to inactivate the S1 nuclease.

Klenow mix

20 mM Tris-Cl, pH 8.0

7 mM MgCl_2

Autoclave.

Ligase mix

10 X ligation buffer	90 μ l
----------------------	------------

T4 ligase	12 μ l
-----------	------------

Sterile water	618 μ l
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Enough for 12 tubes. Use 60 μ l per tube.

B13. DNA sequencing gel mix

A 6% polyacrylamide gel mix was used.

Urea	12.0 g
------	--------

Acrylamide	4.8 g
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Bis acrylamide	0.21 g
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10 X TTE	8.0 ml
----------	--------

Distilled water to	80.0 ml
--------------------	---------

55 ml of the mix was filtered through a 0.8 μ m Millipore filter. TEMED (50 μ l) and 50 μ l of 50% ammonium persulphate was added and mixed before the gel was poured.

B14. Solutions for the preparation of competent *E. coli* cells**TFB1**

100 mM RbCl

50 mM MnCl₂·4H₂O

30 mM potassium acetate

10 mM CaCl₂

15% glycerol

TFB2

100 mM MOPS, pH 7.0

100 mM RbCl

75 mM CaCl₂

15% glycerol

B15. Media

Solid media contained 1.5% (w/v) agar. Media were autoclaved at 121°C for 20 min.

Luria-Bertani medium (LB)

Bacto tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water to	1 l

YPD medium

2% glucose

0.5% peptone

0.3% yeast extract

5 X M9 salts

Na ₂ HPO ₄	30 g
KH ₂ PO ₄	15 g
NaCl	2.5 g
Distilled water to	1 l

The solution was adjusted to pH 7.4 before autoclaving.

***E. coli* M9 minimal media**

5 X M9 salts	200 ml
1 M MgSO ₄	1 ml
0.5% vitamin B1	200 µl
20% glucose	20 ml
5 mM biotin	818 µl
ACC	0.3 g
Distilled water to	1 l

DF salts

KH_2PO_4	0.4 g
Na_2HPO_4	0.6 g
MgSO_4	0.0097 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	100 μg
H_3BO_3	1 μg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.2 μg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	12.5 μg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	7.8 μg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	1.7 μg
Distilled water to	1 l

B16. Antibiotics and media additives

	<u>Concentration</u>	<u>Stock</u>
Ampicillin	100 $\mu\text{l/ml}$	100 mg/ml in water
Kanamycin	50 $\mu\text{l/ml}$	25 mg/ml in water
Rifampicin	200 $\mu\text{l/ml}$	50 mg/ml in DMSO
Spectinomycin	25 $\mu\text{g/ml}$	100 mg/ml in water
Streptomycin	100 $\mu\text{g/ml}$	10 mg/ml in water

Stock solutions of antibiotics dissolved in water were sterilised by filtration through a 0.22-micron filter. All antibiotics were divided into 1 ml aliquots and stored at -20°C .

IPTG (isopropyl-b-D-thio-galactopyranoside)

IPTG	23.4 mg
Distilled water	1.0 ml

The solution was aliquoted and stored -70°C .

X-Gal (5-bromo-4-chloro-3-indolyl-b-galactoside)

X-gal 0.2 g

Dimethylformamide 10 ml

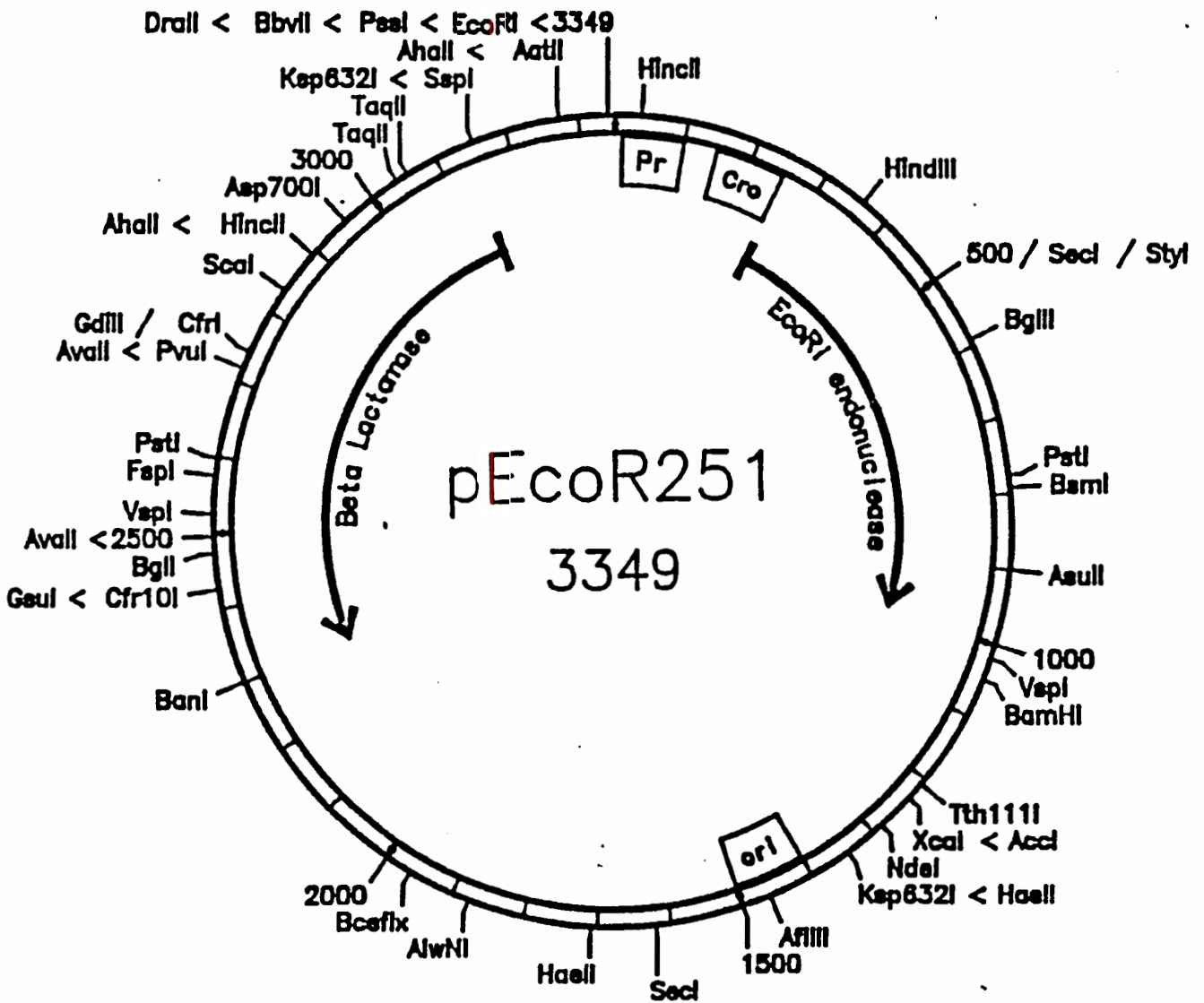
The solution was stored at -70°C.

APPENDIX C

Plasmid maps

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C1.	Restriction endonuclease map of pEcoR251	121
C2.	Restriction endonuclease map of pSK	122



C1. Plasmid map of pEcoR251. DNA is cloned into the gene coding for the restriction endonuclease *EcoRI*. This inactivates the gene thus preventing cell death through the action of the *EcoRI* protein.

ABBREVIATIONS

A	adenine/adenosine
ACC	1-aminocyclopropane-1-carboxylic acid
Amp	ampicillin
ATP	adenosine 5'-triphosphate
bp	base pair
C	cytidine/cytosine
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
°C	degrees centigrade
CsCl	caesium chloride
DIG	digoxigenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
Fig.	figure
g	gravitational acceleration
g	gram
G	guanosine
GCG	Genetics Computer Group
h	hour
kb	kilobase
l	litre
LB	Luria Bertani broth
M	molar
min	minute
ml	milliliter
mol	mole
mRNA	messenger RNA
nm	nanometer
PCR	polymerase chain reaction

RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
Sp	spectinomycin
Str	streptomycin
T	thymidine
T-DNA	Ti-plasmid DNA
TE	tris-EDTA
Ti	tumour inducing
Tris	tris(hydroxymethyl)aminomethane
U	uridine
uv	ultraviolet
<i>vir</i>	virulence
V	volts
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside
α -	alpha
β -	beta
λ -	lambda
μ -	micro

REFERENCES

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1991). Current Protocols. Volume 1. New York: John Wiley and Sons.
- Awad, M. and Young, R.E. (1979). Postharvest variation in cellulase, polygalacturonase, and pectinmethylesterase in avocado (*Persea americana* Mill, cv. Fuerte) fruits in relation to respiration and ethylene production. *Plant Physiol.* 64, 306-308.
- Arshad, M. and Frannkenberger, W.T. (1992). Microbial biosynthesis of ethylene and its influence on plant growth. *Advances in Microbial Ecology* 12, 69-111.
- Balague, C., Watson, C.F., Turner, A.J., Rouge, P., Picton, S., Pech, J. and Grierson, D. (1993). Isolation of a ripening and wound-induced cDNA from *Cucumis melo* L. encoding a protein with homology to the ethylene-forming enzyme. *Eur. J. Biochem.* 212, 27-34.
- Bathgate, B., Purton, M.E., Grierson, D. and Goodenough, P.W. (1985). Plastid changes during the conversion of chloroplasts to chromoplasts in ripening tomatoes. *Planta* 165, 197-204.
- Biggs, M.S., Harriman, R.W. and Handa, A.K. (1986). Changes in gene expression during tomato fruit ripening. *Plant Physiol.* 81, 395-403.
- Bird, C.R., Smith, C.J.S., Ray, J.A., Moureau, P., Bevan, M.W., Bird, A.S., Hughes, S., Morris, P.C., Grierson, D. and Schuch, W. (1988). The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. *Plant Mol. Biol.* 11, 651-662.

Bird, C.R., Ray, J.A., Fletcher, J.D., Boniwell, J.M., Bird, A.S., Teuliers, C., Blain, I., Bramley, P.M. and Schuch, W. (1991). Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. *Biotechnology* 9, 635-639.

Boller, T., Herner, R.C. and Kende, H. (1979). *Planta* 145, 293-303.

Botella, J.R., Schlagnhauser, C.D., Arteca, J.M., Arteca, R.N. and Phillips, A.T. (1993). Identification of two new members of the 1-aminocyclopropane-1-carboxylate synthase-encoding multigene family in mung bean. *Gene* 123, 249-253.

Brady, C.F. (1987). Fruit ripening. *Ann Rev. Plant. Physiol.* 38, 155-178.

Bufler, G. (1984). Ethylene-enhanced 1-aminocyclopropane-1-carboxylic acid synthase activity in ripening apples. *Plant Physiol.* 75, 192-195.

Burg, S.P. and Burg, E.A. (1967). Molecular requirements for the biological activity of ethylene. *Plant Physiol.* 42, 144-152.

Campbell, A.D. and Labavitch, J.M. (1991). Induction and regulation of ethylene biosynthesis and ripening by pectic oligomers in tomato pericarp discs. *Plant Physiol.* 97, 706-713.

Cass, L.G., Kirven, K.A. and Christoffersen, R.E. (1990). Isolation and characterization of a cellulase gene family member expressed during avocado fruit ripening. *Mol. Gen. Genet.* 223, 76-86.

Cervantes, E., Rodriguez and Micolás, G. (1994). Ethylene regulates the expression of a cysteine proteinase gene during germination of chickpea (*Cicer arietinum* L.). *Pl. Mol. Biol.* 25, 207-215.

Chen, S., Jordan, E.M., Wilson, R.B., Draper, R.K. and Clowes, R.C. (1987). Transcription and expression of the exotoxin A gene of *Pseudomonas aeruginosa*. *J. Gen.*

Micro. 133, 3081-3091.

Chetelat, R.T., Klann, E., DeVerna, J.W., Yell, S. and Bennett, A.B. (1993). Inheritance and genetic mapping of fruit sucrose accumulation in *Lycopersicon chmielewskii*. *Plant J.* 4, 643-650.

Christoffersen, R.E., Warm, E. and Laties, G.G. (1982). Gene expression during fruit ripening in avocado. *Planta* 155, 52-57.

Christoffersen, R.E., Tucker, M.L. and Laties, G.G. (1984). Cellulase gene expression in ripening avocado fruit: the accumulation of cellulase mRNA and protein as demonstrated by cDNA hybridization and immunodetection. *Plant Mol. Biol.* 3, 385-391.

Considine, P.J., Flynn, N. and Patching, J.W. (1977). Ethylene production by soil microorganisms. *Appl. Environ. Microbiol.* 33, 977-979.

Crookes, P.R. and Grierson, D. (1983). Ultrastructure of tomato fruit ripening and the role of polygalacturonase isoenzymes in cell wall degradation. *Plant Physiol.* 72, 1088-1093.

Dali, N., Michaud, D. and Yell, S. (1992). Evidence for the involvement of sucrose phosphate synthase in the pathway of sugar accumulation in sucrose-accumulating tomato fruits. *Plant Physiol.* 99, 434-438.

Deikman, J. and Fischer, R.L. (1988). Interaction of a DNA binding factor with the 5'-flanking region of an ethylene-responsive fruit ripening gene from tomato. *EMBO J.* 7, 3315-3320.

DellaPenna, D., Alexander, D.C. and Bennett, A.B. (1986). Molecular cloning of tomato fruit polygalacturonase: analysis of polygalacturonase mRNA levels during ripening. *Proc. Natl. Acad. Sci. USA.* 83, 6420-6424.

- Devereux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12, 387-397.
- Dong, J.G., Kim, W.T., Yip, W.K., Thompson, G.A., Li, L., Bennett, A.B. and Yang, S.F. (1991). Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. *Planta* 185, 38-45.
- Dong, J.G., Olson, D., Silverstone, A. and Yang, S.F. (1992). Sequence of a cDNA coding for a 1-aminocyclopropane-1-carboxylate oxidase homolog from apple fruit. *Plant Physiol.* 98, 1530-1531.
- Dopico, B., Lowe, A., Wilson, I.D., Merodio, C. and Grierson, D. (1993). Cloning and characterisation of avocado fruit mRNAs and their expression during ripening and low-temperature storage. *Pl. Mol. Biol.* 21, 437-449.
- Draper, J., Scott, R., Armitage, P. and Walden, R. (1988). Plant genetic transformation and gene expression - a laboratory manual. Oxford: Blackwell Scientific Publications.
- Dworkin, M. and Foster, J. (1958). Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* 75, 592-601.
- Ealing, P.M. (1994). Lipxygenase activity in ripening tomato fruit pericarp tissue. *Phytochemistry* 36, 547-552.
- Ecker, J.R and Davis, R.W. (1987). Plant defense genes are regulated by ethylene. *Proc. Natl. Acad. Sci. USA* 84, 5202-5206.
- Elliott, K.J., Butler, W.O., Dickinson, C.D., Konno, Y., Vedvick, T.S., Fitzmaurice, L. and Mirkov, T.E. (1993). Isolation and characterization of fruit vacuolar invertase genes from two tomato species and temporal differences in mRNA levels during fruit ripening. *Plant Mol. Biol.* 21, 515-524.

- Enyedi, A. J., Yaipani, N., Silverman, P. and Raskin, I. (1992). Signal molecules plant resistance to pathogenes and pests. *Cell* 70, 879-886.
- Eyal, Y., Meller, Y., Lev-Yadun, S. and Fluhr, R. (1993). A basic-type PR-1 promoter directs ethylene responsiveness, vascular and abscission zone-specific expression. *Plant J.* 4, 225-234.
- Frecknall, E.A and Pattenden, G. (1984). Carotenoid differences in isogenic lines of tomato fruit colour mutants. *Biochemistry* 23, 1707-1710.
- Freifelder, D. (1987). Molecular Biology. Boston: Jones and Bartlett Publishers.
- French, C.S. and Milner, H.W. (1955). Disintegration of bacteria and small particles by high-pressure extrusion. *Methods in Enzymology* 1, 64-67.
- Fukuda, H., Ogawa, T. and Tanase, S. (1993). Ethylene production by micro-organisms. *Adv. Microbial. Physiol.* 35, 275-308.
- Gillapsy, G., Ben-David, H. and Gruissem, W. (1993). Fruits: a developmental perspective. *Plant Cell* 5, 1439-1451.
- Giovannoni, J.J., DellaPenna, D., Bennett, A.B. and Fischer, R.L. (1989). Expression of a chimeric polygalacturonase gene in transgenic *rin* (ripening inhibitor)tomato fruit results in polyuronide degradation but not fruit softening. *Plant Cell* 1, 53-63.
- Gleave, A.P. (1992). A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* 20, 1203-1207.
- Gomez-Lim, M.A., Valdes-Lopez, V., Cruz-Hernandez, A and Saucedo-Arias, L.J. (1993). Isolation and characterization of a gene involved in ethylene biosynthesis from *Arabodopsis thaliana*. *Gene* 217-221.

Good, X., Langhoff, D., Wagoner, W., Kellogg, J., and Bestwick, R.K. (1994). Transgenic tomatoes expressing s-adenosylmethionine hydrolase show reduced ethylene synthesis and modified ripening. In: Abstracts from the Fourth International Congress of Plant Molecular Biology, Amsterdam.

Gray, J., Picton, S., Shabbeer, J., Schuch, W. and Grierson, D. (1992). Molecular biology of fruit ripening and its manipulation with antisense genes. *Plant Mol. Biol.* 19, 69-87.

Grierson, D. and Tucker, G.A. (1983). Timing of ethylene and polygalacturonase synthesis in relation to the control of tomato fruit ripening. *Planta* 157, 174-179.

Grierson, D., Maunders, M.J., Slater, A., Ray, J., Bird, C.R., Schuch, W., Holdsworth, M.J., Tucker, G.A. and Knapp, J.E. (1986a). Gene expression during tomato ripening. *Phil. Trans. R. Soc. Lond. B* 314, 399-410.

Grierson, D., Tucker, G.A., Keen, J., Ray, J., Bird, C.R. and Schuch, W. (1986b). Sequencing and identification of a cDNA clone for tomato polygalacturonase. *Nucleic Acid Res.* 14, 8595-8603.

Grierson, D. (1992). Control of ethylene synthesis and ripening by sense and antisense genes in transgenic plants. *Proc. R. Soc. Edinburgh* 99B, 79-88.

Grierson, D. and Schuch, W. (1993). Control of ripening. *Phil. Trans. R. Soc. Lond. B* 342, 241-250.

Hall, L.N., Tucker, G.A., Smith, C.J.S., Watson, C.F., Seymour, G.B., Bundick, Y., Boniwell, J.M., Fletcher, J.D., Ray, J.A., Schuch, W., Bird, C.R. and Grierson, D. (1993). Antisense inhibition of pectin esterase gene expression in transgenic tomatoes. *Plant J.* 3, 121-129.

Hamilton, A.J., Lycett, G.W. and Grierson, D. (1990). Antisense gene that inhibits

synthesis of the hormone ethylene in transgenic plants. *Nature* 346, 284-287.

Hamilton, A.J., Bouzayen, M. and Grierson, D. (1991). Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. *Proc. Natl. Acad. Sci. USA* 88, 7434-7437.

Harris, W.M. and Spurr, A.R. (1969). Chromoplasts of tomato fruits. II. The red tomato. *Amer. J. Bot.* 56, 380-389.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* 28, 351-359.

Holdsworth, M.J., Schuch, W. and Grierson, D. (1988). Organisation and expression of a wound/ripening-related small multigene family from tomato. *Plant Mol. Biol.* 11, 81-88.

Holtel, A., Marques, S., Mohler, I., Jakubzik, U. and Timmis, K.N. (1994). Carbon source-dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. *J. Bact.* 176, 1773-1776.

Honma, M. and Shimomura, T. (1978). Metabolism of 1-aminocyclopropane-1-carboxylic acid. *Agric. Biol. Chem.* 42, 1825-1831.

Huang, P., Parks, J.E., Rottmann, W.H. and Theologis, A. (1991). Two genes encoding 1-aminocyclopropane-1-carboxylate synthase in zucchini (*Curcubita pepo*) are clustered and similar but differentially regulated. *Proc. Natl. Acad. Sci. USA*. 88, 7021-7025.

Hubbard, N.L., Huber, S.C. and Pharr, D.M. (1989). Sucrose phosphate synthase and acid invertase as determinants of sucrose concentration in developing muskmelon (*Cucumis melo* L.) fruits. *Plant Physiol.* 91, 1527-1534.

Hubbard, N.L., Pharr, D.M. and Huber, S.C. (1990). Role of sucrose phosphate

synthase in sucrose biosynthesis in ripening bananas and its relationship to the respiratory climacteric. *Plant Physiol.* 94, 201-208.

Ince, J.E. and Knowles, C.J. (1986). *Archives of Microbiology* 146, 151.

Innis, M.A. and Gelfand, D.H. (1990). Optimization of PCRs. In: PCR Protocols. Ed: M.A. Innis. Academic Press Inc., p 3-20.

Inouye, S., Gomada, M., Sangodkar, U.M.X., Makazawa, A. and Makazawa, T. (1990). Upstream regulatory sequence for transcriptional activator XylR in the first operon of xylene metabolism on the TOL plasmid. *J. Mol. Biol.* 216, 251-260.

Ish-Horowicz, D. and Burke, J.F. (1981). Rapid and efficient cosmid cloning. *Nuc. Acids Res.* 9, 2989-2998.

Kellog, J, Good, X., Cohen, C., Langhoff, D., Matsumura, W. and Bestwick, R.K. (1994). Regulated expression of s-adenosylmethionine hydrolase using the tomato E4 promoter reduces ethylene synthesis in ripening tomatoes. In: Abstracts from the Fourth International Congress of Plant Molecular Biology, Amsterdam.

Kende, H. (1989). Enzymes of ethylene biosynthesis. *Plant Physiol.* 91, 1-4.

Kende, H. and Boller, T. (1981). Wound ethylene and 1-aminocyclopropane-1-carboxylate synthase in ripening tomato fruit. *Planta* 151, 476-481.

Kieber, J.J, Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family protein kinases. *Cell* 72, 427-441.

Klee, H.J., Hayford, M.B., Krketzmer, K.A., Barry, G.F. and Kishmore, G.M. (1991). Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. *Plant Cell* 3, 1187-1193.

- Klee, H. J. (1993). Ripening physiology of fruit from transgenic tomato (*Lycopersicon esculentum*) plants with reduced ethylene synthesis. *Pl. Physiol.* 102, 911-916.
- Knapp, J., Moureau, P., Schuch, W. and Grierson, D. (1989). Organisation and expression of polygalacturonase and other ripening related genes in Ailsa Craig "Neverripe" and "Ripening Inhibitor" tomato mutants. *Plant Mol. Biol.* 12, 105-116.
- Knee, M., Sargent, J.A. and Daphne, J. (1977). Cell wall metabolism in developing strawberry fruits. *J. Exp. Bot.* 28, 377-396.
- Koncz, C. and Schell, J. (1986). The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* 204, 383-396.
- Lanahan, M.B., Yen, H., Giovannoni, J.J. and Klee, H.J. (1994). The *Never Ripe* mutation blocks ethylene perception in tomato. *Plant Cell* 6, 521-530.
- Lawton, K.A., Potter, S.L., Uknes, S. and Ryals, J. (1994). Acquired resistance signal transduction in *Arabidopsis* is ethylene independent. *Plant Cell* 6, 581-588.
- Lester, D.R., Speirs, J., Orr, G. and Brady, C.J. (1994). Peach (*Prunus persica*) endopolygalacturonase cDNA isolation and mRNA analysis in melting and nonmelting peach cultivars. *Plant Physiol.* 105, 225-231.
- Li, N. and Mattoo, A.K. (1994). Deletion of the carboxy-terminal region of 1-aminocyclopropane-1-carboxylic acid synthase, a key protein in the biosynthesis of ethylene, results in catalytically hyperactive, monomeric enzyme. *J. Biol. Chem.* 269, 6908-6917.
- Li, P., Manom, H., Haser, H., and Roberts, T. (1991). Raf-1: a kinase currently without a cause but not lacking in effects. *Cell* 64, 479-482.

- Liang, X., Abel, S., Keller, J.A., Shen, N.F. and Theologis, A. (1992). The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 89, 11046-11050.
- Lincoln, J.E. and Fischer, R.L. (1988). Regulation of gene expression by ethylene in wild-type and *rin* tomato (*Lycopersicon esculentum*) fruit. *Plant Physiol.* 88, 370-374.
- Lincoln, J.E., Campbell, A.D., Oetiker, J., Rottmann, W.H., Oeller, P.W., Shen, N.F. and Theologis, A. (1993). LE-ACS4, a fruit ripening and wound-induced 1-aminocyclopropane-1-carboxylate synthase gene of tomato (*Lycopersicon esculentum*). *J. Biol. Chem.* 268, 19422-19430.
- Lingle, S.E. and Dunlap, J.R. (1987). Sucrose metabolism in netted muskmelon fruit during development. *Plant Physiol.* 84, 386-389.
- MacDiarmid, C.W.B. and Gardner, R.C. (1993). A cDNA sequence from kiwifruit homologous to 1-aminocyclopropane-1-carboxylic acid oxidase. *Plant Physiol.* 101, 691-692.
- Madhusudhan, K.T., Huang, G., Burns, G. and Sokatch, J.R. (1990). Transcriptional analysis of the promoter region of the *Pseudomonas putida* branched-chain keto acid dehydrogenase operon. *J. Bact.* 172, 5655-5663.
- Manning, K. (1994). Changes in gene expression during strawberry fruit ripening and their regulation by auxin. *Planta* 194, 62-68.
- Martineau, B., Houck, C.M., Sheehy, R.E. and Hiatt, W.R. (1994). Fruit-specific expression of the *A. tumefaciens* isopentenyl transferase gene in tomato: effects on fruit ripening and defense-related gene expression in leaves. *Plant J.* 5, 11-19.
- McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R. and Fraley, R. (1986). Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium*

tumefaciens. *Plant Cell Rep.* 5, 81-84.

McGarvey, D.J., Yu, H. and Christoffersen, R.E. (1990). Nucleotide sequence of a ripening-related cDNA from avocado fruit. *Plant Mol. Biol.* 15, 165-167.

McGlasson, W.B. (1970). The Ethylene Factor. In: Hulme, H.C. (ed.) The biochemistry of fruits and their products. London: Academic Press.

McKeon, T. and Yang, S.F. (1987). Biosynthesis and metabolism of ethylene. In: Davies, P.J. (ed) Plant hormones and their role in plant growth and development. Boston: Martinus Nijhoff.

Miron, D. and Schaffer, A.A. (1991). Sucrose phosphate synthase synthase, and invertase activities in developing fruit of *Lycopersicon esculentum* Mill. and the sucrose accumulating *Lycopersicon hirsutum* Humb. and Bonpl. *Plant Physiol.* 95, 623-627.

Oeller, P.W., Min-Wong, L., Taylor, L., Pile, B.A., Theologis, A. (1991). Reversible inhibition of tomato fruit senescence by antisense RNA. *Nature* 254,437-439.

Palleroni, N.J. (1981). Introduction to the family Pseudomonadaceae. In: Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G. (eds) The prokaryotes. A handbook on habitats, isolation and identification of bacteria. New York: Springer Verlag, p 655-663.

Peck, S.C., Olson, D.C. and Kende, H. (1993). A cDNA encoding 1-aminocyclopropane-1-carboxylate oxidase from pea. *Plant Physiol.* 101, 689-690.

Peever, T.L. and Higgins, V.J. (1989). Electrolyte leakage, lipoxygenase, and lipid peroxidation induced in tomato leaf tissue by specific and nonspecific elicitors from *Cladosporium fulvum*. *Plant Physiol.* 90, 867-875.

Peleman, J., Boerjan, W., Engler, G., Seurinck, J., Botterman, J., Alliotte, T., Van Montagu, M. and Inze, D. (1989). Strong cellular preference in the expression of a housekeeping gene of *Arabidopsis thaliana* encoding S-adenosylmethionine synthetase. *Plant Cell* 1, 81-83.

Penarrubia, L., Aguilar, M., Margossian, L. and Fischer, R.L. (1992). An antisense gene stimulates ethylene hormone production during tomato fruit ripening. *Plant Cell* 4, 681-687.

Pfeiffer, N. (1994). FDA oks Calgene's Flavr Savr™ tomato for marketing in supermarkets in the U.S. *Genetic Engineering News* 14, 1.

Picton, S., Gray, J., Barton, S., AbuBakar, U., Lowe, A. and Grierson, D. (1993a). cDNA cloning and characterisation of novel ripening-related mRNAs with altered patterns of accumulation in the *ripening inhibitor (rin)* tomato ripening mutant. *Plant Mol. Biol.* 23, 193-207.

Picton, S., Barton, S.L., Bouzayer, M., Hamilton, A.J. and Grierson, D. (1993b). Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. *Plant J.* 3, 469-481.

Raven, P.H., Evert, R.F. and Eichhorn, S.E. (1986). Biology of Plants. New York: Worth Publishers.

Ray, J., Knapp, J., Grierson, D., Bird, C. and Schuch, W. (1988). Identification and sequence determination of a cDNA clone for tomato pectin esterase. *Eur. J. Biochem.* 174, 119-124.

Raz, V. and Fluhr, R. (1993). Ethylene signal is transduced via protein phosphorylation events in plants. *Plant Cell* 5, 523-530.

Redgwell, R.J. and Fry, S.C. (1993). Xyloglucan endotransglycosylase activity increases during kiwifruit (*Actinidia deliciosa*) ripening. *Plant Physiol.* 103, 1399-1406.

Rhodes, M.J.C. (1970). The climacteric and ripening fruits. In: Hulme, H.C. (ed.) *The biochemistry of fruits and their products*. London: Academic Press.

Rodrigues-Pousada, R.A., De Rycke, R., Dedonder, A., Van Caeneghem, W, Engler, G., Van Montagu, M. and Van Der Straeten, D. (1993). The *Arabidopsis* 1-aminocyclopropane-1-carboxylate synthase gene 1 is expressed during early development. *Plant Cell* 5, 897-911.

Ross, G.S., Knighton, M.L. and Lay-Yee, M. (1992). An ethylene-related cDNA from ripening apples. *Plant Mol. Biol.* 19, 231-238.

Rottmann, W.H., Peter, G.F., Oeller, P.W., Keller, J.A., Shen, N.F., Hagy, B.P., Taylor, L.P., Campbell, A.D. and Theologis A. (1991). 1-aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. *J. Mol. Biol.* 222, 937-961.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Press.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.

Sanglard, D. (1993). Gopher search.

Sato, T. and Theologis, A. (1989). Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylate synthase, the key enzyme for ethylene biosynthesis in plants. *Proc. Natl. Acad. Sci. USA* 86, 6621-6625.

Sheehy, R.E., Pearson, J., Brady, C.J. and Hiatt, W.R. (1987). Molecular characterization of tomato fruit polygalacturonase. *Mol. Gen. Genet.* 208, 30-36.

Sheehy, R.E., Honma, M., Yamada, M., Sasaki, T., Martineau, B. and Hiatt, W.R. (1991). Isolation, sequence, and expression in *Escherichia coli* of the *Pseudomonas* sp. strain ACP gene encoding 1-aminocyclopropane-1-carboxylate deaminase. *J. Bact.* 173, 5260-5265.

Slater, A., Maunders, M.J., Edwards, K., Schuch, W. and Grierson, D. (1985). Isolation and characterisation of cDNA clones for tomato polygalacturonase and other ripening-related proteins. *Plant Mol. Biol.* 5, 137-147.

Smith, C.J.S., Watson, C.F., Morris, P.C., Bird, C.R., Seymour, G.B., Gray, J.E., Arnold, C., Tucker, G.A., Schuch, W., Harding, S. and Grierson, D. (1990). Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. *Pl. Molec. Biol.* 14, 369-379.

Smith, J.J. and John, P. (1993). Activation of 1-aminocyclopropane-1-carboxylate oxidase by bicarbonate/carbon dioxide. *Biochemistry* 32, 1381-1386.

Smith, J.J., Zhang, Z.H., Schofield, C.J., John, P. and Baldwin, J.E. (1994). Inactivation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase. *J. Exp. Bot.* 45, 521-527.

Spanu, P., Reinhardt, D. and Boller, T. (1991). Analysis and cloning of the ethylene-forming enzyme from tomato by functional expression of its mRNA in *Xenopus laevis* oocytes. *EMBO J.* 10, 2007-2013.

Stolp, H. and Gadkari, D. (1981). Nonpathogenic members of the genus *Pseudomonas*. In: Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G. (eds) *The prokaryotes. A handbook on habitats, isolation and identification of bacteria*. New York: Springer-Verlag.

- Tang, X., Wang, H., Brandt, A.S. and Woodson, W.R. (1993). Organization and structure of the 1-aminocyclopropane-1-carboxylate oxidase gene family from *Petunia hybrida*. *Plant Mol. Biol.* 23, 1151-1164.
- Tanksley, S.D., Young, N.D., Paterson, A.H. and Bonierbale, M.W. (1989). RFLP mapping in plant breeding: new tools for an old science. *Biotechnology* 7, 257-264.
- Theologis, A. (1992). One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. *Cell* 70, 181-184.
- Theologis, A. (1993). What a gas! *Current Biology* 6, 369-371.
- Tigchelaar, E.C., McGlasson, W.B. and Buescher, R.W. (1978). Genetic regulation of tomato fruit ripening. *HortScience* 13, 508-513.
- Trebitsh, T., Goldschmidt, E.E. and Riov, J. (1993). Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme, in *Citrus* fruit peel. *Proc. Natl. Acad. Sci USA* 90, 9441-9445.
- Tucker, G.A., Robertson, N.G., and Grierson, D. (1980). Change in polygalacturonase isoenzymes during the "ripening" of normal and mutant tomato fruit. *Eur. J. Biochem.* 112, 119-124.
- Tucker, G.A., Robertson, N.G., and Grierson, D. (1981). The conversion of tomato-fruit polygalacturonase isoenzyme 2 into isoenzyme 1 *in vitro*. *Eur. J. Biochem.* 115, 87-90.
- Tucker, G.A. and Grierson, D. (1982). Synthesis of polygalacturonase during tomato fruit ripening. *Planta* 155, 64-67.
- Van Der Straeten, D., Van Wiemeersch, L., Goodman, H.M. and Van Montagu, M. (1990). Cloning and sequence of two different cDNAs encoding 1-aminocyclopropane-

1-carboxylate synthase in tomato. *Proc. Natl. Acad. Sci. USA* 87, 4859-4863.

Van Haaren, M.J.J. and Houck, C.M. (1991). Strong negative and positive regulatory elements contribute to the high-level fruit-specific expression of the 2A11 gene. *Plant Mol. Biol.* 17, 615-630.

Van Larebeke, N., Engler, G., Holsters, M., Van den Elsacker, S., Zaenen, I., Schilperoort, R.A. and Schell, J. (1974). Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. *Nature* 252, 169-170.

Viera, J. and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for mutagenesis and sequencing with synthetic universal primers. *Gene* 19, 259-286.

Vioque, B. and Castellano, J.M. (1994). Extraction and biochemical characterization of 1-aminocyclopropane-1-carboxylic acid oxidase from pear. *Physiol. Plant.* 90, 334-338.

Vogeli-Lange, R., Frundt, C., Hart, C.M., Nagy, F. and Meins, F. (1994). Developmental, hormonal, and pathogenesis-related regulation of the tobacco class I β -1,3-glucanase B promoter. *Plant Mol. Biol.* 25, 299-311.

von Heijne, G. (1984). How signal sequences maintain cleavage specificity. *J. Mol. Biol.* 173, 243-251.

Wilson, I.D., Zhu, Y., Burmeister, D.M. and Dilley, D.R. (1993). Apple ripening-related cDNA pAP4 confers ethylene-forming ability in transformed *Saccharomyces cerevisiae*. *Plant Physiol.* 102, 783-788.

Xu, Y., Chang, P.L., Liu, D., Narasimhan, M.L., Raghothama, K., Hasegawa, P.M. and Bressan, R.A. (1994). Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell* 6, 1077-1085.

- Yang, S.F. and Hoffman, N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Ann. Rev. Plant Physiol.* 35, 155-189.
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33, 103-119.
- Yip, W., Dong, J., Kenny, J.W., Thompson, G.A. and Yang, S.F. (1990). Characterization and sequencing of the active site of 1-aminocyclopropane-1-carboxylate synthase. *Proc. Natl. Acad. Sci. USA* 87, 7930-7934.
- Young, E. (1994). Altered tomato faces ban from British shops. *New Scientist* 13 August, 9.
- Zambryski, P. (1988). Basic processes underlying *Agrobacterium* mediated DNA transfer to plant cells. *Ann. Rev. Genet.* 22, 1-30.
- Zambryski, P.C. (1992). Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 43, 465-490.
- Zappe, H., Jones, D.T. and Woods, D.R. (1986). Cloning and expression of a *Clostridium acetobutylicum* endoglucanase, cellobiase and amino acid biosynthesis genes in *E. coli*. *J. Gen. Microbiol.* 132, 1367-1372.